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Functional and association analysis of polymorphisms in selected genes of fat metabolism

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List of abbreviations

AA	arachidonic acid
aa	amino acid
ABL	abetalipoproteinemia
Ala	alanin
apoB	apolipoprotein B
ARP	apolipoprotein A1 regulatory protein
BMD	bone mineral density
BMI	body mass index
CD	cluster of differentiation
C/EBP	CCAAT enhancer binding protein
CEU	CEPH: Utah residents with ancestry from northern and western Europe
CHB+JPT	Han Chinese in Beijing, China and Japanese in Tokyo, Japan
CHD	coronary heart disease
ChiP	chromatin immunoprecipitation
CNV	copy number variation
COUP	chicken ovalbumin upstream promoter
COX	cyclooxigenase
CRP	C-reactive protein
cSNP	coding SNP
CVD	cardiovascular disease
Cys	Cysteine
D6D	delta 6 desaturase
dias	diastolic
DR	down regulator of transcription
EPIC	European Prospective Investigation into Cancer and Nutrition
ER	endoplasmatic reticulum
FA	fatty acid
FABP	fatty acid binding protein
FH	familial hypercholesterolemia
FOXA	forkhead box A
FTO	fat mass and obesity associated gene
EGF	epidermal growth factor
ERR	estrogen related receptor
GATA	TF that binds to the sequence “GATA”
GATE	gamma activated transcriptional element
GBF	GATE binding factor
Gln	Glutamine
GLP	glucagon like peptide
GSH	glutathione
GWA	genome wide association study
H	Histidine
His	Histidine
HCV	hepatitis C virus
HDL	high density lipoprotein
HNF	hepatocyte nuclear factor

HOMA	homeostatic model assessment
htSNP	haplotype tagging SNP
I	Isoleucine
IDL	intermediate density lipoprotein
IFN	interferon
IL	interleukin
IMT	intima media thickness
IRS	insulin response element
irs	insulin resistance syndrome
KORA	Kooperative Gesundheitsforschung in der Region Augsburg
LD	linkage disequilibrium
LDL	low density lipoprotein
LRH	liver receptor homolog
LTA	lymphotoxin-alpha
MAPEG	membrane associated proteins in eicosanoid and glutathione metabolism
MCR	melanocortin receptor
MetS	metabolic syndrome
MICK	Metabolic Intervention Cohort Kiel
mRNA	messenger ribonucleic acid
MTTP	microsomal triglyceride transfer protein
MUFA	monounsaturated FA
NASH	non-alcoholic steatohepatitis
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
OCT	octamer-binding TF
OGTT	oral glucose tolerance test
PAD	peripheral arterial disease
pat	patients
PDI	protein disulfide isomerise
PG(E, G, H)	prostaglandin (E, G, H)
PGC	PPAR gamma coactivator
pp	post prandial
PPAR	peroxisome proliferator-activated receptor
PTGES	prostaglandin E synthase
PUFA	polyunsaturated FA
PYY	peptide tyrosine tyrosine
Q	Glutamine
R	Arginine
ROBO	roundabout axon guidance receptor homolog
ROS	reactive oxygen species
Rosi	Rosiglitazone
RXR	retinoid X receptor
SCPSB	Scotland Coronary Prevention Study
SD	standard deviation
SFA	saturated fatty acids
SHP	small heterodimer partner

SMAD	SMA (*C. elegans*) and mothers against decapentaplegic (*D. melanogaster*) homolog
SMRT	silencing mediator of retinoid and thyroid receptor
SNP	single nucleotide polymorphism
Sp	specificity protein
SREBP	sterol regulatory binding protein
STAT	signal transducer and activator of transcription
T	Threonine
T2D	type 2 diabetes mellitus
TCF7L	transcription factor 7-like
TF	transcription factor
TFA	trans fatty acids
TFH	hypothetical HeLa cell specific TF
TG	triglyceride
TGF	transforming growth factor
Thr	Threonine
TNF	tumor necrosis factor
UTR	untranslated region
VLDL	very low density lipoprotein
YRI	Yoruba in Ibadan, Nigeria

Summary

The genes encoding fatty acid binding protein 2 (FABP2), microsomal triglyceride transfer protein (MTTP) and prostaglandin E synthase 2 (PTGES2) are involved in the regulation of the metabolism of dietary fat. Variants of the FABP2, MTTP and PTGES2 genes are associated with traits of the metabolic syndrome (MetS). This work expands the knowledge about the influence of the 3 gene loci on diverse MetS traits via association analysis in the KORA cohort with more than 8000 participants. Further, functional analysis of the FABP2 promoter was carried out in cell culture regarding a PPARγ/RXRα regulation.

FABP2 mediates intestinal absorption and cytosolic transport of non-esterified long chain free fatty acids (FA) as well as their secretion into triglyceride rich chylomicrons. Further, FABP2 transports FA into the nucleus as ligands for transcription factors (TF). The FABP2 promoter comprises polymorphisms c.-80_-79insT, c.-136_-132delAGTAG, c.-168_-166delAAGinsT, c.-260G>A, c.-471G>A and c.-778G>T, which result in two haplotypes A and B. Studies in relatively small cohorts provide preliminary evidence that FABP2 promoter haplotypes are associated with type 2 diabetes (T2D) and BMI.
Here, the influence of haplotypes on BMI was examined via linear regression analysis of 8072 male and female participants of the KORA cohort. A reduction of -0.39 BMI units (p=0.02) in homozygous male FABP2 promoter haplotype B carrier was found. Carrier of haplotype B (AB+BB) showed a significant decrease in BMI of -0.19 BMI units (p=0.03). In accordance, a significant reduction in BMI of the minor haplotype carrier in the BMI point categories of 25-30 (-0.10 BMI units, p=0.03) and <30 (-0.37 BMI units, p=0.02) were found. Female carriers showed no association with BMI.
Functional analysis of haplotypes by Rosiglitazone stimulated PPARγ/RXRα displays a 2-fold higher activity of haplotype B than A. As shown by chimeric FABP2 promoter constructs, the higher responsiveness of FABP2 haplotype B is mainly but not solely determined by polymorphism c.-471G>A. As shown by electro mobility shift assays and promoter reporter assays, OCT1 interacts with the -471 region of FABP2 promoters, induces the activities of both FABP2 promoter haplotypes and abolishes the different activities of haplotypes induced by Rosiglitazone stimulated PPARγ/RXRα.

MTTP is a key regulator in the assembly and secretion of chylomicrons and VLDL in the intestine and in liver. Associations between MTTP variants and traits of the MetS are carried

out in relatively small cohorts and are not consistent. Seven htSNPs covering a 52 kb region of the MTTP locus and two cSNPs (I128T, H297Q) were analysed for MetS trait alteration via linear regression in 7582 participants of the KORA study cohort. A MTTP haplotype containing the minor allele of H297Q showed a significant decrease of -0.64 BMI units (p=0.04) in females but not in males. Only females carrying the Q-allele showed a decrease in BMI (Q-carrier: -0.26 BMI unit, p=0.02; Q/Q: -0.66 BMI units, p=0.01), waist circumference (Q-carrier: -0.66 cm, p=0.01; Q/Q -1.44 cm, p=0.01) and total cholesterol (Q-carrier: -0.05 mmol/l, p=0.03; Q/Q: -0.11 mmol/l, p=0.03). Males revealed no association with any of the three measured traits of the MetS.

PTGES2 is required for production of the antilipolytic compound prostaglandin E_2. The H-allele of the R298H polymorphism in the PTGES2 gene was associated with lower risk of T2D. To explore a PTGES2 gene influence on BMI alteration, the R298H SNP and three htSNPs covering a 20 kb gene region were analysed in the KORA study cohort with 8079 participants. A statistically significant difference in BMI between the heterozygous PTGES2 R298H genotype and the homozygous R/R genotype was found in males but not in females. Males with the R/H genotype showed a decrease in BMI of -0.30 BMI units (p=0.02) in comparison to R/R males. A haplotype comprising the minor allele of PTGES2 R298H showed a significant decrease of -0.19 BMI units in males (p=0.04) but not in females. Other haplotypes and single htSNPs were not significantly associated with BMI.

Association analysis in the KORA cohort with more than 8000 participants revealed association between BMI and polymorphisms of the FABP2 promoter and the MTTP and PTGES2 genes. The MTTP SNP H297Q and related haplotypes are also associated with waist circumference and total cholesterol. Functional analysis exhibited, that the FABP2 promoter haplotype B is 2-fold more inducible by PPARγ/RXRα than haplotype A. This difference is caused by polymorphism c.-471G>A. In addition, OCT1 was identified as a TF regulating the FABP2 promoter. In conclusion, variants of the FABP2, MTTP and PTGES2 genes, which are involved in fat metabolism, contribute to the development of the MetS.

Zusammenfassung

Das Fettsäure-Bindungsprotein 2 (FABP2), das mikrosomalen Triglycerid-Transfer Protein (MTTP) und die Prostaglandin E Synthase 2 (PTGES2) sind beteiligt an der Regulierung des Fettstoffwechsels. Polymorphismen in den Genen vom FABP2, MTTP und PTGES2 sind mit Markern des Metabolischen Syndroms (MetS) assoziiert. Diese Arbeit erweitert das Wissen über den Zusammenhang zwischen diesen 3 Gen Bereichen und Markern des MetS auf der Basis von Assoziationsstudien in der KORA Kohorte mit mehr als 8000 Teilnehmern. Weiterhin wurde die Regulation des FABP2 Promotors durch PPARγ/RXRα funktionell in einem Zellkulturansatz untersucht.

FABP2 reguliert die Absorption und den zytosolischen Transport von nicht veresterten langkettigen freien Fettsäuren (FS) sowie deren Einbau in Chylomikronen im Darm. Weiterhin transportiert FABP2 FS in den Zellkern, in dem diese als Liganden für Transkriptionsfaktoren fungieren. Der FABP2 Promotor zeigt die Polymorphismen c.-80_-79insT, c.-136_-132delAGTAG, c.-168_-166delAAGinsT, c.-260G>A, c.-471G>A und c.-778G>T, die in den Haplotypen A and B resultieren. Studien in kleinen Kohorten zeigten Assoziationen der FABP2 Promotor Haplotypen mit Diabetes Typ 2 (T2D) und BMI. Lineare Regressions-Analysen der beiden Haplotypen im Bezug auf den BMI von 8072 Männern und Frauen der KORA Kohorte offenbarte eine Reduktion um -0.39 BMI Einheiten (E.) (p=0.02) von homozygoten männlichen FABP2 Promotor Haplotyp B-Trägern. Generell hatten Männer mit dem Haplotyp B einen um -0.19 E. (p=0.03) signifikant erniedrigten BMI. Übereinstimmend hatten Träger des seltenen Allels eine BMI Erniedrigung in den Gruppen mit einem BMI von 25-30 (-0.10 E., p=0.03) und <30 (-0.37 E., p=0.02). Weibliche Träger des seltenen Allels zeigten keine signifikanten Assoziationen. Funktionelle Untersuchungen der FABP2 Promotor Haplotypen nach PPARγ/RXRα Transfektion führten zu einer 2-fach erhöhten Aktivität des Haplotyps B im Vergleich zu A im Zellkulturansatz. FABP2 Promotor Chimären zeigten, dass die stärkere Aktivierbarkeit des Haplotyps B wesentlich von dem c.-471G>A SNP abhängig ist. Elektro Mobility Shift Assays und Promotor Reporter Analysen zeigten eine Promotor aktivierende Bindung von OCT1 im Bereich des c.-471 SNP. Die haplotypen-spezifische Induktion durch PPARγ/RXRα wurde dadurch aufgehoben.

MTTP ist ein Schlüsselenzym in der Bildung und der Sekretion von Chylomikronen und VLDL im Darm und in der Leber. Assoziationsstudien zwischen MTTP SNPs und Markern

des MetS, besonders Lipid-Werten, wiesen in kleinen Kohorten widersprüchliche Ergebnisse auf. 7 htSNPs, sowie 2 cSNPs (I128T, H297Q), die eine 52 kb Region des MTTP Locus umspannen, wurden auf ihr MetS Marker veränderndes Potential mittels Linearer Regression in 7582 Teilnehmern der KORA Kohorte hin untersucht. Ausschließlich weibliche Träger eines Haplotyps mit dem seltenen Allel des H297Q SNP zeigten eine signifikante Erniedrigung des BMIs um -0.64 E. (p=0.03). Ausschließlich Frauen mit einem Q-Allel zeigten Verminderungen des BMIs (Q- Träger: -0.26 BMI E., p=0.02; Q/Q: -0.66 BMI E., p=0.01), des Hüftumfangs (Q- Träger: -0.66 cm, p=0.01; Q/Q -1.44 cm, p=0.01) und des gesamt Cholesterins (Q- Träger: -0.05 mmol/l, p=0.03; Q/Q: -0.11 mmol/l, p=0.03). Männliche Träger des Q-Allels wiesen keine Veränderung dieser Parameter auf.

PTGES2 bildet das antilipolytische Prostaglandin E_2. Das H-Allel des R298H SNP im PTGES2 Gen ist mit einem erniedrigten T2D Risiko assoziiert. Um den Einfluss von PTGES2 SNPs auf BMI Veränderungen zu untersuchen, wurden der R298H SNP und 3 htSNPs, welche eine 20 kb Region des PTGES2 Gens umspannen, in der KORA Kohorte mit 8079 Teilnehmern analysiert. Ein statistisch signifikanter Unterschied zwischen BMI und heterozygoten Trägern des R298H SNP im Vergleich zu Trägern des häufigen Allels wurde ausschließlich in Männern gefunden. Träger des R/H Genotyps wiesen eine Reduktion um -0.30 BMI E. (p=0.02) auf. Männer die einen 298H Haplotyp aufwiesen zeigten einen um -0.19 E. erniedrigten BMI (p=0.04). Weiteren htSNPs oder Haplotypen zeigten keine Assoziationen zu einem veränderten BMI.

Lineare Regressions-Analysen in der KORA Kohorte (n>8000) zeigten Assoziationen zwischen dem BMI und Polymorphismen im FABP2 Promotor und den Genen des MTTP und der PTGES2. Der MTTP H297Q SNP ist weiterhin mit Veränderungen im Hüftumfang und des Gesamt Cholesterins assoziiert. Funktionelle Analysen des FABP2 Promotors zeigten, dass die 2-fach höhere Aktivierbarkeit des Haplotyps B durch PPARγ/RXRα durch den SNP c.-471G>A hervorgerufen wird. Zusätzlich wurde OCT1 als ein Transkriptionsfaktor identifiziert, der den FABP2 Promotor reguliert. Zusammenfassend kann gesagt werden, dass Polymorphismen in den Genen des FABP2, MTTP und PTGES2, die für den Fettstoffwechsel wichtig sind, zur Entstehung des MetS beitragen.

General introduction

The metabolic syndrome (MetS) encompasses the most prevalent diseases in the western world including obesity, glucose and insulin disturbances, dislipidemia and high blood pressure [1]. These traits are accompanied by cardiovascular disease (CVD) and type 2 diabetes mellitus (T2D). T2D incidence is projected to double to 350 million cases by the year 2030 [2, 3]. With the completion of the Human Genome Project in 2003 [4] and the International HapMap Project in 2005 [5] the possibility to identify genetic variants associated with MetS was considerably enhanced resulting in numerous candidate gene approaches. Many candidates associated with MetS traits in initial studies could not be confirmed in further populations. This multitude of (unverified) candidate genes is unlikely to be solely false positives. In fact, especially the disease-associations of polymorphisms in small cohorts strongly depend on a variety of gene-gene and/or gene-environment interactions like food composition [6].

The aim of this work was to expand the knowledge about the effects of selected polymorphisms in fat metabolism genes previously associated with traits of MetS and T2D. Therefore more than 8000 participants were genotyped in the KORA cohort for variants in the fatty acid binding protein 2 (FABP2) promoter, the gene loci of the microsomal triglyceride transfer protein (MTTP) and the prostaglandin E synthase 2 (PTGES2). Further functional analysis of the FABP2 promoter was carried out in cell culture regarding a PPARγ/RXRα regulation.

The fatty acid binding protein 2 (FABP2) promoter

The FABP family was first described 1982 after isolation from rat liver [7]. With its function of hydrophobic molecule binding the FABP family belongs to the calycin superfamily and is conserved in vertebrates and invertebrates [8]. A more precise classification of the vertebrate cytoplasmic FABPs was made, according to the tissue in which their expression was first detected, into liver FABP (L-FABP or FABP1), intestinal FABP (I-FABP or FABP2), heart FABP (H-FABP or FABP3), adipocyte FABP (A-FABP or FABP4), epidermal FABP (E-FABP or FABP5), ileal FABP (Il-FABP or FABP6), brain FABP (B-FABP or FABP7), myelin FABP (MP2 or FABP8) and testis FABP (T-FABP or FABP9). Nonetheless, their expression is not necessarily restricted to their eponymous tissue [9].

Despite their differences in protein sequence, the molecular weight of 14 to 15 kDa and the tertiary structure are features shared by all family members. As illustrated in Fig. 1 the protein forms a twisted β-barrel surrounding a hydrophobic core, which is composed of 10 antiparallel β-strands, which are organized into 2 β-sheets oriented nearly orthogonally [10]. One end of the β-barrel is capped by a small helix-turn-helix motif. Within the β-barrel a large water-filled cavity lined with polar and hydrophobic amino acids (aa) is responsible for sequestering the carboxylate groups of fatty acids (FA) [11]. The α-helical N-terminus is mandatory for membrane interaction and therefore for overall FA transfer. Apart from the FABP classification mentioned above, these proteins can be grouped according to their binding abilities. Group 1 (FABP1 and 6) is capable of binding FA and bulky ligands such as bile acids, cholesterol and haem. Group 2 (FABP3-5 and 7-9) binds FA, retinoids and eicosanoids. Only FABP2 solely binds FA, although in a bent instead of U-shaped conformation like groups 1 and 2 [12].

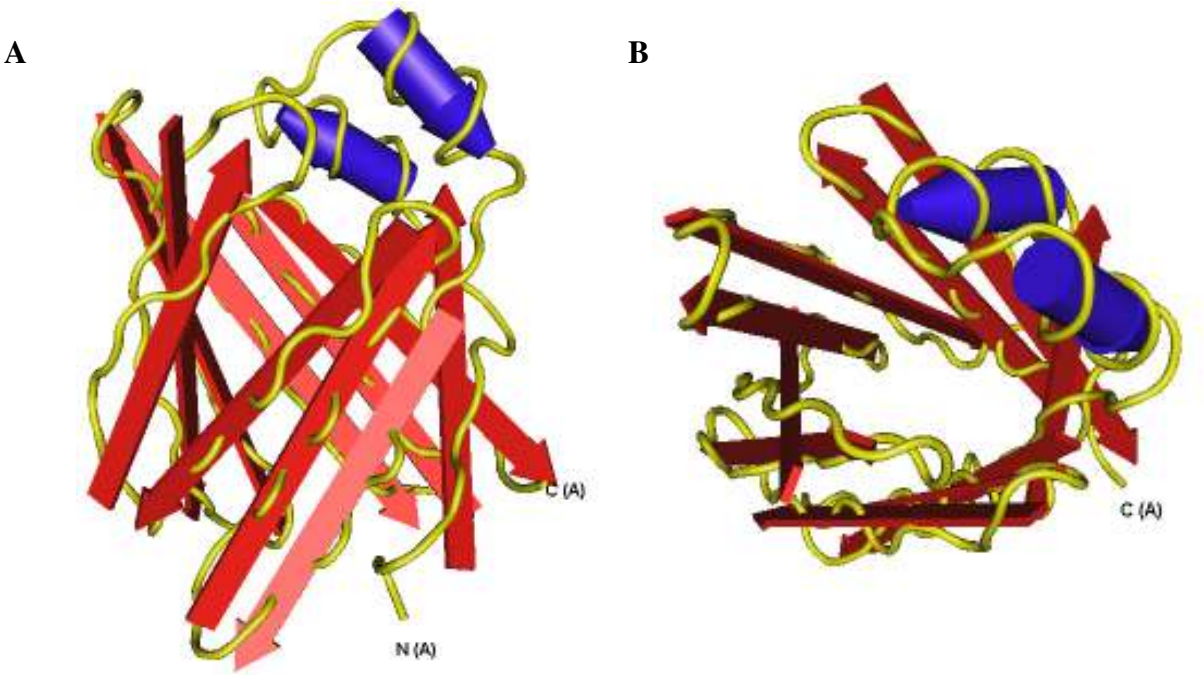

Figure 1. 3D structure of the FABP2 protein viewed from two different angles (A, B). Taken from the NCBI structure database viewed via CN3D viewer version 4.1. Red indicates β-strands, blue indicates α-helixes, arrowheads indicate direction [13].

Besides passive diffusion across plasma membranes, FA translocation can also be enhanced by the fatty acid transport protein (FATP) and plasma bound $FABP_{(PM)}$ [14]. The FA dissociation from the plasma membrane into the cytoplasm is increased by cytosolic FABPs, which enhance the aqueous solubility of FA [15]. In addition to FABP-mediated increase in desorption and cytoplasmic diffusion, FA can interact directly with the phospholipid bilayer

of acceptor membranes [16, 17]. Besides their function in lipid metabolism, FABPs are involved in FA-mediated gene regulation via modification, binding and activation of transcription factors (TF), modifying mRNA stability and influencing TF expression [18]. One of the most frequently mentioned TF family associated with FA regulation are the peroxisome proliferator-activated receptors (PPAR) as their regulative potential can be influenced by FABPs [19-21].
Furthermore, FABPs are relevant for cell growth and proliferation in different tissues [9]. In addition, FABP1 showed antioxidative potential in transfected liver cell lines [22].

The human FABP2 gene (OMIM accession number: 134640; GenBank: NM_000134.3) is located on chromosome 4q28-q31, contains 4 exons and codes for a 15.1 kDa protein comprising 132 aa [23].
FABP2 is expressed in the small intestine together with FABP1 and 6, whereas FABP2 is solely found intestinally [24, 25]. The highest FABP2 expression is quantified in the jejunum and decreases in the proximal duodenum and colon [23]. Maximum protein abundance is detected in the fat absorbing upper villus region, while FABP2 is not detectable in crypt cells [26]. The expression of FABP2 is up-regulated simultaneously with cell and tissue differentiation [26, 27]. Its expression is increased by FA, hormones and cytokines (e.g. butyric acid and oleic acid, insulin, leptin, phosphatidylcholine, hydrocortisone, tumor necrosis factor α (TNFα) and peptide tyrosine tyrosine (PYY)), and decreased after exposure to epidermal growth factor (EGF) in cell culture [28-30]. Mouse studies revealed an increase of FABP2 mRNA after administration of PPARα agonists [31]. Knock out (KO) of transforming growth factor β 1 (TGFβ1) or estrogen related receptor α (ERRα) showed down-regulation of FABP2 [32]. ERRα regulation may indicate a sex specific gene expression pattern of FABP2 as seen in FABP2 KO mice. The FABP2 promoters of rat and mouse showed *cis*-binding sites for a CCAAT enhancer binding protein (C/EBP), hepatocyte nuclear factor 4 (HNF4) and the apolipoprotein A1 regulatory protein 1 (ARP1) [33]. Influence of the TF TGFβ-SMAD/GATA4 on FABP2 promoter activity was also shown in mice [34]. Recently, regulation of the FABP2 promoter via the TFs HNF1α and HNF4α was revealed in cell culture [35, 36].

FABP2 localization is congruent with its postulated function in absorption, cellular binding and trafficking of non-esterified long-chain free FA, which are incorporated into triglyceride (TG)-rich chylomicrons for subsequent transport [8, 37, 38]. Although FABP2 seems to be

the most important protein of its family in the gut the intestinal expression of FABP1 is 5-fold higher compared to FABP2 [39]. This may be due to their different activities. The transport of FAs is markedly faster via FABP2 than via FABP1, caused by different mechanisms of interaction with acceptor model membranes. Transfer by FABP2 is mediated by protein-membrane collisional interactions while FAs have to diffuse through the aqueous phase for FABP1 binding. This is due to altered integrity of the α-helix of FABP2, which is mandatory for membrane interaction (see above) [40].

The redundancy of FABP1, 2 and 6 in intestinal cells may contribute to the inconsistent results from *in vivo* and *in vitro* studies according to FABP2 function. FABP2 overexpression in endogenously FABP1 and 2 expressing Caco-2 cells led to reduced fatty acid incorporation and secretion but to increased cellular lipid content [29, 41]. Overexpression in human intestinal epithelial cell line (HIEC-6) enhanced mitochondrial FA oxidation, cholesterol transport gene expression and influenced interaction with nuclear receptors [42]. Undifferentiated ES cells stably expressing FABP2 revealed elevated initial and maximal FA uptake and cytoplasmatic diffusion [43]. L-cell fibroblasts expressing FABP2 also exhibited increased cytoplasmatic diffusion and initial FA esterification into TG as well as elevated TG and cholesteryl ester mass [44, 45]. FABP2 KO in mice revealed altered lipid metabolism in male animals only [32]. They showed traits of the MetS such as increased body weight, elevated plasma insulin and TG. They also showed high fat diet-induced fatty liver although altered FABP1 level were not observed [46, 47]. At this, differences in FABP1 and 2 expressions between mice and humans have to be considered. While FABP2 expression is similar, human FABP1 transcript levels are 2.5-fold higher [39].

The FABP2 gene carries a G>A exchange at codon 54 (rs1799883), resulting in an Ala-allele and a Thr-allele [48]. This polymorphism has been analysed extensively with divergent results. The Thr54 allele has been associated with a variety of traits of the MetS: i) especially those concerning the FA metabolism, such as reduced HDL-cholesterol, elevated total and LDL-cholesterol, hypertriglyceridemia, increased affinity for long-chain FA, FA flux into the circulation and FA oxidation [49-52]; ii) concerning the carbohydrate metabolism, such as insulin resistance, increased fasting plasma glucose and fasting insulin [53-55]; iii) and concerning inflammation and complex diseases like elevated C-reactive protein (CRP) and interleukin 6 (IL6) level, obesity, T2D, CVD, stroke, reduced resting metabolic rate and renal diseases [56-59]. In contrast, a number of studies showed no association of the Thr54 allele with changes in BMI, total and LDL-cholesterol, hypertriglyceridemia, FA absorption rate,

lipid or glucose metabolism, insulin resistance, T2D or CVD [50, 58, 60-65]. Third, some studies revealed beneficial effects of the Thr54 polymorphism like reduced total and LDL-cholesterol, reduced total abdominal adipose tissue, lower subcutaneous abdominal fat and glucose levels, decreased systolic blood pressure and a later onset of alcoholic cirrhosis [66-69]. Porcine FABP QTLs were initially also linked to elevated fat mass [9] while recent studies with physically mapped markers stated that the FABP cluster was outside this region [70]. Divergent results may be caused by different ethnic, phenotypic and lifestyle backgrounds of study populations, with varying frequencies of the Ala54Thr polymorphism between 18% and 40% [53].

Association between FABP2 Thr54 genotypes and reduced insulin sensitivity was increased after normalisation for differences in body composition and habitual physical activity (PA) levels. Another study showed association of the Thr54 SNP with reduced insulin sensitivity only when individuals were consuming a high fat diet [71]. Interaction between the Thr54 allele, fat composition and consumption was uncovered by Dworatzek et al. They showed that Thr54 carrier had increased chylomicron cholesterol but only after administration of olive oil [72]. However, Morcillo et al. stated that the Thr54 polymorphism was associated with insulin resistance in subjects with a high intake of sunflower oil [73]. Normolipidemic Thr-carrier showed increased fasting free FA after a saturated FA (SFA) diet in contrast to homozygote Ala-carrier [74]. Furthermore, Thr-carrier had lower glucose tolerance, lower insulin action and higher lipid oxidation rates even if they were following a low fat diet [75]. Obese children with the Thr54 polymorphism displayed lower arachidonic acid (AA) and delta-6-desaturase (D6D) activity in fasting plasma [76]. AA is a pro-inflammatory stimulus, but the positive effect of low AA levels on inflammation faces the negative effect of low AA levels in PPARγ signalling [77], since AA can act as a ligand for PPARγ, which leads to increased insulin sensitivity [78, 79]. Other authors explained the effects on AA and D6D with the type of dietary FA ingested and not with the Ala54Thr polymorphism [73].

Functional analysis in Caco-2 cells transfected with FABP2 bearing the Thr-allele showed increased cellular FA transport and TG secretion compared to the Ala-allele [8]. Human intestinal explants homozygous for the Thr-allele showed elevated secretion of newly esterified TG and chylomicrons versus Ala-allele carrier [37].

Since the FABP2 Ala54Thr polymorphism has been analysed extensively this work focuses on the analysis of the FABP2 promoter and its polymorphisms in order to reveal a more

precise knowledge about the interaction between FABP2, lipid metabolism and traits of the MetS.

Sequencing studies of the 5´ upstream promoter region of FABP2 showed insertion/deletion sites c.-80_-79insT (rs5861422), c.-136_-132delAGTAG (rs5861423) and c.-168_-166delAAGinsT (rs1973598) as well as SNPs c.-260G>A (rs6857641), c.-471G>A (rs2282688) and c.-778G>T (rs10034579). These six variants are in complete linkage disequilibrium (LD) resulting in only two haplotypes A>B with an allele frequency of 44% in Caucasians [80-82]. The FABP2 promoter haplotype B revealed a protective effect against traits of the MetS. It was associated with reduced BMI, lower postprandial TG levels and decreased risk for T2D [80, 81, 83]. Prior association studies were carried out in relatively small cohorts composed of 100-1000 participants. The first of four papers of this work addresses the association of the FABP2 promoter polymorphisms and BMI as a main trait of the MetS in a large (n=8072) population-based survey of the KORA study cohort.

→ {1} M. Böhme, H. Grallert, M. Klapper, C. Gieger, A. Fischer, I. Heid, H.-E. Wichmann, F. Döring, T. Illig, **Association between functional FABP2 promoter haplotypes and body mass index: Analyses of 8072 participants of the KORA cohort study**, *Molecular Nutrition and Food Research* 53 (2009).

Functional analysis of haplotypes showed almost 2- to 3-fold lower transcriptional activity of the rare haplotype B compared to haplotype A in Caco-2 cells [83]. This activity difference is due to decreased binding affinity of GATA5 and 6 to the c.-80_-79insT polymorphism [84]. Recently, a 2-fold higher activity of haplotype B compared to haplotype A was detected by Rosiglitazone stimulated PPARγ/RXRα in FABP2 deficient HeLa cells [84, 85]. Accordingly, the second paper of this work investigates the functional regulation of the FABP2 promoter by Rosiglitazone stimulated PPARγ/RXRα in different cell lines.

→ {2} M. Böhme, I. Nitz, F. Döring, M. Klapper, **Analysis of the transcriptional regulation of the FABP2 promoter haplotypes by PPARgamma/RXRalpha and OCT1**, *Biochim Biophys Acta* 1779 (2008) 616-21.

The Microsomal Triglyceride Transfer Protein (MTTP)

The human microsomal triglyceride transfer protein (MTTP, OMIM accession number: 157147; GenBank: NM_000253.2) is located on chromosome 4q24, contains 19 exons and codes for a 97 kDa protein comprising 876 aa residues. It belongs to a family of large lipid transfer proteins with orthologs in nematodes, fish, insects and mammals [86]. MTTP comprises four structural domains, three β-sheets (N, A, C) and one α-helix. The N-sheet and the α-helix are relevant for binding with the N-terminus of apolipoprotein B (apoB), while the A- and C-sheet form the lipid transfer/binding domain. Kinetic studies suggest that MTTP has two lipid-binding sites. The first site is probably involved in lipid transfer while the second site may have a function in membrane association. The membrane association domain, which is relevant for lipid droplet formation, consists of N- and A-sheet parts. MTTP affinity for apoB increased after lipid association, which implements interdependent domain activities [87]. MTTP encodes the large subunit (M subunit) of the heterodimeric microsomal triglyceride transfer protein (MTP). Protein disulfide isomerase (PDI, P subunit) completes the heterodimer, which plays a central role in lipid transport and lipoprotein assembly.

MTTP is abundantly expressed in the liver and intestine. Other tissues, including hematopoietic cells, synthesize less MTTP due to an alternative promoter and exon 1 (MTTPv1). The exon variation encodes an N-terminal change of 2 aa, while functional differences were not observed. The abundance of MTTP in non-apoB-expressing tissues seems to be relevant for CD1d activation [88]. However, in adipocytes MTTPv1 is primarily localized to the Golgi and therefore may have a distinct role in lipid storage in adipose tissue [89]. The MTTP promoter region contains binding sites for the overlapping insulin response element (IRS)/sterol regulatory binding proteins (SREBP) and the COUP-TFII. Both TFs promote down-regulation. The MTTP promoter is activated by HNF4α, which is essential for hepatic expression, HNF1α, LRH1, DR1, as well as PPARα/RXRα and FOXA2. In addition to direct interaction of these TFs with *cis*-regions on the MTTP promoter, regulation via co-factors and environmental influences was described. PPARγ coactivator 1β (PGC1β) enhanced transcription via FOXA2 and RXRα, while the small heterodimer partner (SHP) down-regulates MTTP via HNF4α and LRH1 interaction [90].

While MTP displays orthologs in multiple kingdoms their protein function differs between invertebrates and vertebrates [86]. MTP functions in vertebrates are i) lipid transfer activity and membrane binding, ii) lipid droplet formation in the endoplasmatic reticulum and iii)

apoB binding [91]. During lipid transfer MTP interacts with donor and acceptor vesicles for acquisition and delivery. The optimal lipid transfer activity was measured in the presence of neutrally charged membranes. Presence of negatively charged lipids in membranes decreased the lipid transfer activity of MTP [92]. In hepatocytes MTP was crucial for aggregation of lipid droplets and their transport from the cytosol into the Golgi and endoplasmatic reticulum (ER). Further, TG content in microsomes was MTP dependent, while phosphatidylcholine concentration was independent of MTP [93, 94]. MTP is an essential chaperon for nucleation and desorption of primordial apoB-containing lipoproteins with a density under 1.063 g/ml, namely chylomicrons, LDL and VLDL [90].
MTP is involved in immune system signalling via lipidisation of CD1d, which is a major histocampatibility complex class 1 homolog presenting lipid and glycoplipid antigens to natural killer T cells. MTP directly transfers phospholipids to the antigen binding cleft of CD1d in the ER and thereby facilitates rapid cytokine production [95].

The impact of polymorphisms on MTTP promoter activity and protein function were analysed comprehensively via association analysis although with contradictory results. Table 1 summarises association studies between SNPs of the MTTP locus and metabolic traits. The polymorphisms studied most extensively are the promoter SNPs c.-493G>T (rs1800591), c.-400A>T (rs1800803), c.-164T>C (rs1800804) and the coding SNP I128T (rs3816873). These SNPs are summarised based on their nearly complete linkage disequilibrium [96]. In different study populations they were associated with reduced, increased or no traits of the MetS. Functional analysis of the 128T polymorphism showed reduced thermal stability and increased cleavage of MTTP by proteolysis, which indicates a less compact fold and structural stability. This impairs apoB binding and, consequently, decreases LDL production. Accordingly, a protective effect of the 128T polymorphism and the promoter SNPs is postulated [97]. In contrast, a SREBP1a regulation of the MTTP gene via SNP c.-164T>C results in a higher activity of the rare C-allele. This promoter activity was synergistically up-regulated by the rare c.-493G>T polymorphism [98]. Furthermore, three common missense polymorphisms (Q95H (g.343G>C), Q244E (rs17599091), H297Q (rs2306985)) were described which exhibited no influence on MetS [96]. Another study comprising 9 patients with high apoB level and 7 controls were genotyped for polymorphisms in the MTTP gene. 4 out of 11 SNPs were associated with increased abdominal visceral adipose tissue and LDL-apoB. These were the previously described -400T allele, and the homozygote carrier of the new 282G/G, 933C/C and 1151A/A alleles [99].

Table 1. Associations between MTTP SNPs and metabolic traits

polymorphism	*traits*	*reference*
493G>T (rs1800591)	- **reduced** total and LDL-cholesterol, VLDL, apoB, TG (only in FH women), IDL and LDL production rate, smaller LDL particle size, carotic IMT, pp-insulin, dias. blood pressure, T2D	middle-aged white American men [100], FH pat. [101], white UK men [96, 102], visceral obese and hyperinsulinemic Chinese men [103], Chinese TD2 pat. [104], T2D pat. [105], healthy German volunteers [106], MICK+EPIC [107]
c.- 400A>T (rs1800803) c.- 164T>C (rs1800804)	- **increased** lipid content of VLDL, total and LDL-cholesterol, TG, FFA, apoB, apoB-48, intrahepatic TG (in T2D), plasma insulin, BMI, NASH, CHD, PAD, BMD, fibrosis, resistin and CRP, fall of HDL-C and apoA1, steatosis (HCV3-pat)	middle-aged white American men [100], Chinese TD2 pat. [104], CARDIA [108], T2D pat. [109], white American men [96, 110], NASH pat. [111, 112], SCPSB [113], Austrian PAD pat. [114], Japanese women [115], Chinese [116], Italian [117], Italian HCV3 pat. [118]
I128T (rs3816873)	- **no** association with plasma lipoproteins (total-, LDL-, HDL-cholesterol and their lipid mass), TG, apoB and AI, IRS, CHD, steatosis (HCV-pat)	white American men [110], myocardial infarction pat. [119], Framingham Offspring study [120], SCPSB [113], French-Canadian youth [62], HCV-pat. [121]
Q95H (g.343G>C)	- **increased** longevity	nonagenarians [122]
	- **no** change in lipids, lipoproteins and anthropometric variables, longevity (meta-analysis)	white UK men [96, 123], nonagenarians [124]
Q244E (rs17599091)	- **no** change in lipids, lipoproteins and anthropometric variables	white UK men [96, 123]
H297Q (rs2306985)	- **no** change in lipids, lipoproteins and anthropometric variables	white UK men [96, 123]

Abbreviations used: BMD: bone mineral density, CARDIA: African American men, CHD: coronary hart disease, CRP: C-reactive protein, dias: diastolic, FH: familial hypercholesterolemia, HCV: hepatitis C virus, HCV3: hepatitis C virus genotype 3, IMT: intima media thickness, IRS:: insulin resistance syndrome, NASH: nonalcoholic steatohepatitis, PAD: peripheral arterial disease, pat: patients, pp: postprandial, SCPSB: Scotland Coronary Prevention Study, T2D: type 2 diabetes mellitus

The same group found an association between a homozygous c.419-420insA and the dietary fat absorption deficiency abetalipoproteinemia (ABL) in a family study [125].
Expression studies in cell culture with truncated C-terminus MTTP abolished TG transfer and deletion of a C-terminal 20 aa residue resulted in ABL [126].

Since the initial association studies were carried out with small cohorts, the third paper of this work investigates the association between MTTP variants and the MetS traits BMI, waist circumference and total cholesterol in the large KORA cohort with 7582 participants. For comprehensive locus coverage 8 haplotype-tagging SNPs (rs3811800, rs3816873, rs1057613, rs2035816, rs982424, rs2306985, rs2903202, rs1491244) comprising 52kb of the MTTP locus were examined.

→ {3} M. Böhme, H. Grallert, A. Fischer, C. Gieger, I. Nitz, I. Heid, C. Kohl, H.-E. Wichmann, T. Illig, F. Döring, **MTTP variants and body mass index, waist circumference and serum cholesterol level: Association analyses in 7582 participants of the KORA study cohort**, *Molecular Genetics and Metabolism* 95 (2008) 229-232..

The Prostaglandin E Synthase 2 (PTGES2)
The prostaglandin E synthase 2 gene isoform 1 (PTGES2, OMIM accession number: 608152; GenBank: NM_025072.4) maps to the chromosomal band 9q34.11, contains 9 exons and codes for a 33 kDa protein comprising 377 aa residues. It is required for prostaglandin E_2 (PGE_2) production from PGH_2. PGE_2 is the most common prostanoid of the eicosanoid family. It is synthesised from endogenously produced or exogenically supplied semi-essential AA, which is converted to PGG_2 and PGH_2 by cyclooxigenases (COX1 and 2). PGE_2 exerts a broad range of bioactivities like smooth muscle dilatation/contraction, sodium excretion, body temperature regulation, induction of pain, inflammation-associated bone resorption and inhibition of immune responses [127]. Beside its considered role as the principal prostaglandin in acute inflammation, PGE_2 also blocks lipolysis and induces hypertrophy in adipose tissue. Furthermore, leptin releasing properties were described [128].

Three PTGES are known. The first is the ubiquitously and constitutivly expressed cytosolic (c)PTGES, which is relevant for basal PGE_2 production. The second is the inducible microsomal or membrane bound (m)PTGES1, which plays a central role in inflammation.

Due to this function, mPTGES1 is the PTGES analysed most extensively [129]. Both PTGES belong to the membrane-associated proteins involved in eicosanoid and glutathione (GSH) metabolism (MAPEG) family, unlike the third PTGES. The third (m)PTGES2 does not require GSH for activation, although its activity is enhanced by GSH and other thiol compounds. Based on sequence analysis PTGES2 showed a ^{110}Cys-x-x-Cys^{113} region, which is also present in thioredoxin and glutarredoxin. Since PTGES2 activity was abrogated by ^{110}Cys mutation, it can be assigned to the thioredoxin family [130].

Three PTGES2 isoforms are known, of which the isoforms 1 is the most abundant. It is constitutively expressed in various tissues, including skeletal muscle, kidney, liver and adipocytes, were PTGES1 levels are low [131]. Unlike PTGES1, PTGES2 is not induced by the pro-inflammatory cytokines IL-1β, TNFα or NF-κB. PTGES2 promoter studies on mice showed multiple Sp1 sites and a GC box without a TATA box motif [132]. Interestingly, KO of the inflammatorily stimulated COX2 gene in mice decreased PTGES2 expression, while PTGES1 expression was unaffected [133]. In an animal model of adjuvant-induced arthritis PTGES1 and COX2 level increased, while cPTGES and COX1 level were not altered, as described prior for inflammatory diseases. The PTGES2 level slightly decreased [134]. This indicates a more basal metabolic function of PTGES2 that is not primarily inflammation-dependent.

PTGES2 is identical with the gamma activated transcriptional element (GATE) binding factor (GBF1), which is induced by interferon γ (IFNγ). Since IFNγ is a critical cytokine in viral infection, PTGES2 may still be involved in disease-related metabolism. Accordingly, PTGES2 is up-regulated in a variety of cancers. However, the strong *trans*-activating activity of GBF1 is accompanied by a weak DNA binding property [135].

Association studies between PTGES2 and MetS traits are limited. Linkage between the PTGES2 harbouring chromosomal band 9q34.11 and body weight has been described [136]. Three independent study populations showed associations between the PTGES2 R298H (rs13283456) polymorphism and reduced risk for T2D [137-139]. The promoter SNP g.-417G>T and three haplotype tagging SNPs (rs884115, rs10987883, rs4837240) showed no association with traits of the MetS [138]. Association between the R298H and reduced fasting insulin, pp-insulin after OGTT and reduced HOMA index disappeared after adjustment for BMI, though BMI was only marginally associated with the H-allele [137].

The fourth paper of this work examines if the rare H-allele contributes to BMI changes. Therefore, the association between PTGES2 R298H polymorphism and BMI in a large (n=8079) population-based survey of the KORA study cohort was analysed.

→ {4} A. Fischer, H. Grallert, M. Böhme, C. Gieger, I. Lindner, I. Heid, H.-E. Wichmann, F. Döring, T. Illig, **Association analysis between the PTGES2 R298H polymorphism and body mass index in 8079 participants of the KORA study cohort**, *Genetic Testing* (2008).

General discussion

Genotypic association of the FABP2 promoter haplotypes with BMI

Few studies have yet carried out association analysis of the FABP2 promoter and traits of the MetS. The first paper of this work explores the association of the FABP2 promoter haplotypes with BMI, a predominant component of the MetS. All previous studies were conducted in small cohorts with an average size of 150 and never more than 1300 people. For the present study, the KORA cohort with 8072 participants was used for association examination.

No association between the FABP2 promoter haplotypes and BMI was found in the study population unstratified by gender and in the female subgroup. Male subjects showed a significant reduction in BMI of -0.39 BMI units (p=0.02) for the homozygous FABP2 promoter haplotype B compared with homozygous haplotype A. Male carrier of haplotype B (additive model) showed a significant decrease in BMI of -0.19 BMI units (p=0.03). These findings were congruent with a significant reduction in BMI of the minor haplotype carrier in the BMI point categories of 25-30 (-0.10 BMI units, p=0.03) and <30 (-0.37 BMI units, p=0.02).

Significant haplotype differences were only seen after gender stratification and solely for male participants. A reduction in BMI of male haplotype B carrier is further evidence for the previously stated protective effects of the FABP2 promoter haplotype B against traits of the MetS and T2D [80, 81, 83]. Although these results are in contrast to increased BMI in female and lack of association with male BMI of non-Hispanics from Colorado (USA) [81]. These differences may partly be due to different diet composition, especially FA, as described for the FABP2 Ala54Thr polymorphism [72, 73]. Medication is not surveyed in the KORA cohort. This may confound results since pharmacologic treatment potentially influences traits of the MetS [140]. Additionally, divergent associations of the FABP2 Ala54Thr SNP and the FABP2 promoter polymorphisms in various populations may be due to a varying LD [81]. The LD (D' and r^2) between the FABP2 promoter haplotype tagging SNP c.-471G>A and the Ala54Thr polymorphism were analysed [unpublished] using Haploview 4.0 [141]. Table 2 shows D' and r^2 for the 3 HapMap supported populations CEU (CEPH: Utah residents with ancestry from northern and western Europe), YRI (Yoruba in Ibadan, Nigeria) and CHB+JPT (Han Chinese in Beijing, China and Japanese in Tokyo, Japan) [142]. Haploview analysis revealed maximal LD scores only for the CEU population and solely with the D' algorithm. Calculations with the r^2 algorithm showed no complete LD in any population of the HapMap

project. The complete LD in CEU was not confirmed by a study of Formanack and Baier, who found complete LD exclusively in Pima Indians but not in white Americans [82].

Table 2. D' and r^2 between the FABP2 promoter haplotype tagging SNP c.-471G>A and the FABP2 Ala54Thr polymorphism

population	*D'*	*r^2*
CEU	1.000	0.491
YRI	0.926	0.267
CHB+JPT	0.910	0.559

Abbreviations used: CEU: (CEPH) population from Utah residents with ancestry from northern and western Europe, YRI: population from Yoruba in Ibadan, Nigeria, CHB+JPT: population from Han Chinese in Beijing, China and Japanese in Tokyo, Japan

A gender specific phenotype has previously been described in FABP2 KO mice [32], which was shown to be associated with elevated body weight, plasma insulin and TG, as well as high fat diet induced fatty liver specifically in male mice [46, 47]. It must be admitted that this is in contrast to reduced BMI of the *in vitro* less active FABP2 haplotype B.

The finding that FABP2 is associated with BMI solely in males is not implausible, since different sets of genes influence BMI in men and women. Various studies have shown estrogen- and testosterone-specific gene regulation. This highlights the impact of age as a confounding factor in association studies. Indeed, a strong age-dependent association with obesity has been shown for the gene roundabout axon guidance receptor homolog 1 (ROBO1) in several studies [143]. Besides, anatomical and physiological differences between men and women in fat distribution, insulin resistance, lipid metabolism and cardiovascular function have been described. These gender-dependent variations can result in a sex-specific phenotype [144], albeit gender-dependent and haplotype specific FABP2 regulation awaits to be functionally examined.

One possible regulative pathway may act via PPARγ. Paper {2} shows increased haplotype B activity after Rosiglitazone stimulated PPARγ/RXRα administration. Animal models revealed higher PPAR activity in male mice compared to females [145, 146]. This may lead to a larger activity range of ectopic expressed FABP2 haplotype B, which results in a more distinctive effect on metabolic traits.

A positive effect of increased haplotype B activity may be the regulation of the glucagon like peptide 1 (GLP1). FABP2 may not be expressed solely in enterocytes but also in GLP-expressing intestinal epithelial L-cells [Klapper, M, personal communication]. Despite its role in insulin und glucagon homeostasis GLP1 increases satiety [147]. Carriers of a more active

FABP2 haplotype are able to transport higher amounts of FA, which increase GLP1 expression resulting in earlier satiety. A gender-specific influence may exist, since GLP1 levels are lower in males [148]. Hence, haplotype-specific regulation may have more impact on low male GLP1 levels than on high female levels. In future studies FABP2 and GLP1 expression in genotyped males and females has to be analysed for validation of these hypotheses.

Paper {1} revealed a reduced BMI for non-obese but not for obese males carrying haplotype B. Hence, the FAPB2 promoter haplotype B has a protective effect against overweight only as long as obesity has not occurred.
Men with increased BMI had more intra-abdominal adipose tissue than visceral adipose tissue compared to females [149]. Intra-abdominal fat is associated with increased plasma TG, fasting glucose, postprandial insulin, free FA and blood pressure, as well as with an increase in pro-inflammatory cytokines like TNFα, NF-κB or IL6 [149-151]. Obesity is further associated with an increase in the LDL atherosclerosis marker lipoprotein(a) in men but not in women [152]. The increase in several metabolic risk factors in obese men may abolish the positive effect of the FABP2 haplotype B observed in normal weight men.

In conclusion, the first paper of this work shows association of the FABP2 promoter haplotype B with reduced BMI but only in normal weight males.

Functional analysis of FABP2 promoter haplotypes
Several studies which have analysed the distribution of polymorphisms found that functional SNPs occur in 30-60% of human promoters. They tend to cluster in close proximity, around 100 bp upstream, to the transcription start site. These data indicate that promoters may represent a major site where polymorphisms contribute to human disease [153].
Search for polymorphisms in the FABP2 promoter revealed six polymorphic sites altering the transcriptional activity of FABP2, which were associated with traits of the MetS [80, 81, 83]. Genome wide linkage scan revealed an association of the FABP2 locus with physical fitness in dizygotic female twins [154] but lacked MetS association [155-157]. A 2- to 3-fold lower activity of FABP2 promoter haplotype B in post-confluent Caco-2 cells is caused by decreased binding affinity of GATA5 and 6 to the c.-80_-79insT polymorphism [84]. Additionally, FABP2 deficient HeLa cells exhibited a 2-fold higher activity of haplotype B

than A by Rosiglitazone stimulated PPARγ/RXRα [85]. Here, a PPARγ binding site at position -623/-607 of the FABP2 promoter was identified via *in silico* analysis and electro mobility shift assays (EMSA). Since haplotype specific binding of PPARγ to the FABP2 promoter was not observed this *cis*-acting element for PPARγ can not solely account for haplotype specific regulation. Interaction with one or more TFs seems likely.
Polymorphism c.-471G>A was involved in determining different FABP2 promoter haplotype activities. However, reversal of the mutation in the opposite haplotype did not abrogate PPARγ inductivity. More precise investigation of the region comprising polymorphism -471 via *in silico* and EMSA analysis revealed an octamer-binding TF 1 (OCT1) binding site. Transfection of OCT1 induced FABP2 promoter activity by a factor of three without haplotype specific variation. The different FABP2 promoter haplotype activities after Rosiglitazone stimulated PPARγ/RXRα transfection were abolished after co-transfection with OCT1. Thus, high concentration of OCT1 can override the effect of PPARγ/RXRα on different activities of FABP2 promoter haplotypes.

PPARγ promotes the expression of FABP1 and 4 [158], albeit the relevance for Rosiglitazone dependent PPARγ/RXRα regulation of the FABP2 promoter haplotypes has to be considered carefully. *In vivo* FABP2 is present in the small intestine [23] where PPARα and PPARδ, but not PPARγ, are predominantly expressed [159]. The higher activating potential of FABP2 promoter haplotype B by PPARγ might result in an ectopic expression of FABP2 in PPARγ expressing tissues of haplotype B carriers. PPARγ was associated with T2D and obesity [155-157]. Therefore, FABP2 may exhibit its influence on the MetS via PPARγ specific ectopic regulation.

The ubiquitously expressed OCT1 plays a central role in metabolic homeostasis [160, 161]. However, the OCT1 dependent up-regulation of FABP2 promoter constructs in HeLa cells could not be confirmed in intestinal cells. Transfection of OCT1 in Caco-2 cells showed no effect on FABP2 mRNA level. Even if OCT1 seems not to be relevant for intestinal FABP2 expression, it may contribute to an ectopic expression of FABP2 in tissues with high OCT1 expression like prostate, cortex or lymphocytes [25]. A interdependence between PPARγ/RXRα and OCT1 can be present either via direct binding of RXR with OCT1 [162], as well as via induction of OCT1 expression by PPARγ [163].

Figure 2 discloses a working model for the interaction between TFs and the FABP2 promoter in HeLa cells. PPARγ binds at position -623/-607 and drives haplotype specific activation via interaction with a hypothetical HeLa cell specific TF (TFH1). TFH1 binds to the promoter region around SNP c.-471G>A in a haplotype specific manner. The complex is stabilised by RXRα and Rosiglitazone (Rosi). This results in haplotype specific promoter assay activity (Fig. 2A). Co-transfection with OCT1 abolishes haplotype specific TFH1 binding. Since OCT1 increased neither FABP2 mRNA levels {2} nor luciferase promoter plasmid activity in Caco-2 cells [unpublished], PPARγ/RXRα/OCT1 derived activity in Hela cells seems to be regulated by another factor (TFH2) (Fig. 2B).

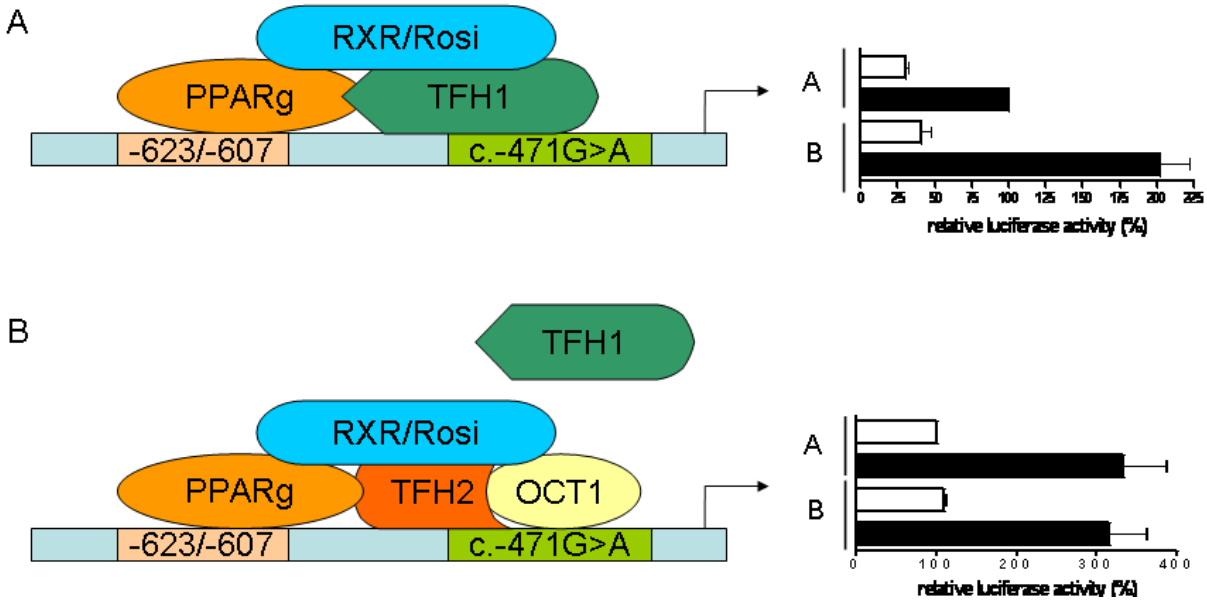

Figure 2. Working model for the HeLa cells specific TF binding to FABP2 promoter haplotypes A and B after transfection with Rosiglitazone (Rosi) stimulated A) PPARγ/RXRα and B) PPARγ/RXRα/OCT1. TFH1/2 abbreviates hypothetical HeLa cell specific TFs. White bars indicate basal activity of luciferase promoter constructs. Black bars show promoter activity after induction with Rosi. N=3, +SD. Refer to text for detailed explanation and to {2} for transfection procedures.

Identification of TFH1 and 2 candidates is not trivial. TFH1 has to be a TF interacting with PPARγ and/or RXRα and competing with OCT1 for binding sites. PPARγ activity and interaction is diverse, ranging from ligand independent transcriptional enhancer function to inhibition of gene activity, incorporated in a multifactor complex comprising several co-factors and ligands. One TFH1 candidate may be NF-κB, since PPARγ interacting properties, as well as overlapping binding sites with OCT1 were described for the CRP promoter [159, 164]. Overlapping with an OCT1 binding site and PPARγ co-factor activity was also shown for AP1 on the IL5 promoter [165]. Interestingly, basal NF-κB and AP1 activity in HeLa cells

was enhanced by TNFα administration [166]. This may indicate an enhanced ectopic activity of FABP2 haplotype B in diseases associated with an increased inflammation status, leading to an adverse feedback effect. FA induce NF-κB and TNFα via elevated ROS production [167]. Increased NF-κB and TNFα may enhance FABP2 expression which elevates FA transport. At this, it has to be considered that PPARγ negatively influences the production of inflammatory cytokines such as TNFα and may slow down the self-enhancing FA-ROS-cytokine-FABP2-FA loop [159]. However, a putative up-regulation of the FABP2 haplotype B in inflammation may be in contrast to a higher haplotype A activity in healthy individuals anticipated from expression in Caco-2 cells. This has to be taken into account when recruiting patients for association studies.

Furthermore, the silencing mediator of retinoid and thyroid receptor (SMRT) regulates transcription of the oxidised LDL receptor together with PPARγ/RXRα. SMRT may be a candidate for TFH2, since binding of OCT1 with SMRT was shown in COS-7 cells. A second candidate interacting with PPARγ and OCT1 is the ubiquitously expressed C/EBPβ [159]. So far, SMRT [168] and C/EBPβ [169] have been described as transcriptional repressors, which is contrary to the activation seen in HeLa cells. Additionally, C/EBPβ may be a TFH1 candidate, since transcriptional interference of OCT1 and C/EBPβ was described for the androgen receptor [170]. Another interesting TFH2 candidate may be the ubiquitously expressed and in cancer up-regulated signal transducer and activator of transcription 3 (STAT3). Promoter assays revealed that the α2-macroglobulin promoter is activated only in the presence of STAT3, even if an enhanceosome, which comprises OCT1 transactivation, is associated with the promoter region [171].

In conclusion, paper {2} revealed haplotype specific activation of the FABP2 promoter by PPARγ. A PPARγ binding site at position -623/-607 was identified. Haplotype specific activity is controlled via polymorphism c.-471G>A. This polymorphic site revealed no PPARγ but OCT1 binding. Even if OCT1 induces the FABP2 promoter, it lacks haplotype specific activity. These effects were solely observed in HeLa cells but not in Caco-2 {2} and HepG2 cells [unpublished].

To elucidate the haplotype specific regulation of the FABP2 promoter via PPARγ/RXRα and OCT1 ChIP analysis should be carried out for both haplotypes in HeLa and Caco-2 cells to reveal TFH1 and 2 candidates. Furthermore, it would be interesting to measure FABP2 mRNA and protein abundance in intestinal and adipocyte biopsies from homozygous haplotype A and B carriers for clarification of putative ectopic expression.

Genotypic association of the MTTP H297Q polymorphism with BMI, waist circumference and total cholesterol

More than four million SNPs have been indentified in coding and non-coding regions of the human genome, occurring roughly every 100 to 1000 bp [153, 172]. To account for genetic variety of the MTTP locus, the third paper of this work displays association analysis with BMI, waist circumference and total cholesterol of 7 htSNPs and 2 non-synonymous cSNPs of a 52 kb MTTP locus region in the KORA cohort.

Multiple studies showed associations of the c.-493G>T, c.-400A>T, c.-164T>C and I128T SNPs and diverse traits of the MetS leading to contradictory results [96]. All studies were conducted in small cohorts with no more than several hundred participants. The KORA cohort with 7582 MTTP genotyped participants exhibits no association with the I128T polymorphism and BMI, waist circumference or plasma lipids. Functional analyses of the promoter polymorphisms and the I128T SNP displayed a combination of a high promoter activity with low protein functionality and vice versa [97, 107]. The resulting consequence of these opposing effects seems to be a neutral phenotype.

Surprisingly, the hitherto inconspicuous H297Q polymorphism revealed association to MetS traits. Stratification for gender revealed that the general reducing effect of the Q-allele in BMI (Q-carrier: -0.18 BMI unit, p=0.01; Q/Q: -0.43 BMI units, p=0.01), waist circumference (Q-carrier: -0.45 cm, p=0.01; Q/Q: -0.99 cm, p=0.01) and total cholesterol (Q-carrier: -0.04 mmol/l, p=0.03; Q/Q: -0.10 mmol/l, p=0.01) is caused by alterations in the female subgroup. Only females carrying the Q-allele showed a decrease in BMI (Q-carrier: -0.26 BMI unit, p=0.02; Q/Q: -0.66 BMI units, p=0.01), waist circumference (Q-carrier: -0.66 cm, p=0.01; Q/Q: -1.44 cm, p=0.01) and total cholesterol (Q-carrier: -0.05 mmol/l, p=0.03; Q/Q: -0.11 mmol/l, p=0.03).

In silico analysis of the His to Gln exchange at position 297 with Protean (DNAstar-Lasergene 7.0) revealed a slightly less charged and more hydrophobic MTTP protein [unpublished]. Position 297 is located in a domain relevant for lipid binding. This suggests increase in lipid acquisition which may lead to faster chylomicron and VLDL maturation in Q-allele carrier. This is consistent with observations of fat malabsorption, hepatic steatosis and insulin resistance in MTTP loss of function patients and KO mice [93, 96].

Conversely, a decrease in delivery and, hence, a retarded lipoprotein maturation is conceivable. This hypothesis may be more congruent with reduced total cholesterol levels. Cholesterol is transported to the periphery via chylomicrons, VLDL and LDL, whereby

MTTP is relevant for the assembly of these lipoproteins [173]. A second function of MTTP is lipid transport. A delay in TG transport may lead to decreased delivery of TG to the periphery especially the abdominal adipose tissue resulting in reduced waist circumference and overall BMI.

Associations with the MTTP 297Q allele were solely found in women. A female-specific more active rare -493T showed lower TG, VLDL, LDL levels and blood pressure [101, 174, 175]. Contrary, 128T allelic T2D men from the EPIC study were associated with reduced waist circumference and BMI. This was not observed in women from the EPIC cohort [107]. Since MTTP and apoB are forming a complex, protein interactions have to be considered. It has been shown that apoB mRNA and protein levels are higher in females [176, 177]. Hence, H297Q dependent MTTP activity may exhibit more pronounced effects in females than in males resulting in a larger phenotype change.

Based on functional associations, the c.-493G>T, c.-400A>T, c.-164T>C and I128T SNPs might have an overall neutral effect. This work revealed a strong impact of the H297Q SNP on lipid metabolism. Hence, it is debateable if former associations were caused by the H297Q polymorphism due to random linkage, rather than evoked by the I128T or promoter SNPs. This would explain contradictory study results mentioned above.
Future studies should analyse H297Q haplotype specific MTTP levels in males and females to reveal functional impact of this polymorphism.
In conclusion, the third paper of this work shows a new association of the MTTP H297Q polymorphism with a strong MetS phenotype. Female Q-carrier showed lower BMI, waist circumference and total cholesterol. Accordingly, re-analysis of prior associated loci can still be important to find new candidates for complex diseases.

Genotypic association of the PTGES2 R298H polymorphism with BMI

Prior studies revealed association of the PTGES2 R298H polymorphism with T2D. To elucidate a BMI mediated influence on T2D the R298H and 3 htSNPs comprising a 20 kb region of the PTGES2 gene were analysed in 8079 participants of the KORA cohort.
No association to BMI was found for the 3 htSNPs as well as for females heterozygous for the 298H allele. Only males heterozygous for the R298H polymorphism showed association with reduced BMI (R/H: -0.30 BMI units, p=0.02). This was confirmed by haplotype analysis.

Only a 298H containing haplotype showed a significant decrease of BMI in males (-0.19 BMI units, p=0.04) but not in females. Homozygous H-allele carrier displayed no association.

A protective effect of the rare H-allele was described for T2D, impaired glucose tolerance, fasting and postprandial insulin levels, HOMA indices and insulin resistance [137-139]. The fourth paper stated a BMI lowering effect of the H-allele but solely in heterozygous males.
Hypothesis generation for a H-allele dependent phenotype remains challenging, since the functions of PGE_2 are diverse. PTGES2 forms homotetramers for enzymatic activity. The R298H SNP is located in the homotetramer forming region which may result in altered enzymatic activity [130]. A heterozygous genotype might lead to a slight decrease in overall PTGES2 function causing a small reduction of PGE_2 level. PGE_2 possesses anti-lipolytic activity and induces adipocyte hypertrophy, hence a reduced PGE_2 level may result in a lower BMI [178-180]. Assuming that PTGES2 homotetramer formation is abolished or highly impaired in homozygous H-allele carrier, the lack of BMI reduction may be due to (over)compensatory up-regulation of other PTGES enzymes. Indeed, PGE_2 level were 2-fold increased in male but not in female homozygous H-allele carrier. However, a heterozygous phenotype showed no reduction of PGE_2 [181].

Gender dependent haplotype specific activity of PTGES2 is not reported until now. It was stated that PTGES genes are regulated via PPARγ [182]. A sex specific expression profile of PPARγ was shown earlier [145]. Further, a putative estrogen binding site in the PTGES2 promoter was examined via *in silico* analysis [181] while promoter studies are lacking.

The IFNγ induced *trans*-activating activity of PTGES2 might be influenced by the R298H polymorphism [135]. Promoter studies with both haplotypes showed only a low activating potential without a haplotype specific pattern [181].

Even if the H-allele exhibits only a small impact on overall obesity and BMI, it may contribute to a risk reduction of T2D and traits of the MetS. Future studies should analyse the gender-specific expression of the PTGES genes for the various haplotypes.
In conclusion, the fourth paper revealed that the PTGES2 R298H SNP is associated with lower BMI in males.

Outlook

Understanding the functional regulation of complex outcomes is demanding as seen for the FABP2 promoter haplotypes. Often different studies reveal opposing results. In addition to candidate gene approaches current research focuses on genome-wide association studies (GWA) to find genes with strong disease association [183]. The most consistently identified genes associated with MetS traits via GWA were transcription factor 7-like 2 (TCF7L2) for T2D [184], the fat mass and obesity associated gene (FTO) for obesity [185] and the pro-inflammatory cytokine lymphotoxin-alpha (LTA) for CVD [186]. One of the most discussed candidate genes associated with several traits of the MetS are PPARγ [187-189] as well as the melanocortin 4 receptor (MCR4) [190].

The emerging field of GWA studies failed to show association between FABP2, MTTP or PTGES2 and traits of the MetS [184-189]. To address these apparent contradictory results, one has to look more carefully at GWAs. First, investigated traits like obesity are considered as "complex" but GWAs often associate only one SNP or cluster with specific "complex" traits. Second, assuming that these SNPs are the genetic markers with the strongest impact, they should be replicated in other GWAs. Indeed, this was only feasible for a few SNPs in diverse traits. Third, the functionality of the associated gene has to be taken into account since the most extreme p-value alone is not an adequate predictor of true effects [191]. And fourth, genes identified by GWAs tend to exert the largest effects, but account only for 5-10% of the trait variance. The majority will be more difficult to identify. This becomes apparent on traits like blood pressure which failed to show GWA associations [190].

Beyond, GWAs are far from representing the whole genome. Analysis of 3 current genome wide chips, 500k (Affimetrix) and HumanHap300/550 (Illumnia), revealed low coverage especially for singleton SNPs. Singleton SNPs are SNPs that can not be predicted by other SNPs via linkage analysis and therefore expanding the chip costs disproportionately. Singleton SNPs are significantly more frequent non-synonymous, splicing site, 5' or 3'UTR than intronic or intergenic SNPs. This ascribes them a potentially higher functional importance. The tested arrays account only for ~50% of known singleton SNPs. [172]. New GWA arrays incorporate copy number variations (CNV) in their design, since CNV contribute considerably to disease traits. Admittedly, currently available arrays miss at least 20% of known CNVs [192]. With decreasing chip costs this coverage will increase in the future but has to be considered when interpreting published results.

With lower costs and higher datasets of GWAs, data analysis will play the most decisive role in the future. GWAs reveal many candidates associated with slight phenotype changes, which

are *per se* significant, while after multiple testing these candidates are declared as false positives. A GWA of 6148 Sardinians showed association with obesity of the FTO gene but not of FABP2, MTTP or PTGES2. A more detailed examination of the dataset revealed association of FABP2 with BMI (-0.10 BMI units, p=0.03), hip circumference (-0.11 cm, p=0.01) and weight (-0.08 kg, p=0.03) prior to multiple testing. MTTP was analysed but showed no significant associations, while PTGES2 was not examined [193]. Hence, GWAs give more insights into genetic associations, but their results still have to be carefully studied. Further, lack of prior associations in GWA between FABP2 and MetS traits are not contradictory, since most GWAs barely account for gender stratification [144]. In accordance, paper {1} shows association only in the male subgroup but not within the whole study cohort.

Table 3. Association analysis of FABP2 promoter polymorphism and the MTTP H297Q SNP with BMI

Genotype		*Frequencies (n)*			*Unit change in BMI in kg/m^2 [95% confidence interval] (p values)*		
FABP2 promoter	MTTP Q297H	All	Men	Wom.	All	Men	Women
11	11	1052	515	537	1	1	1
12+22	11	2049	1055	994	0.04 [-0.28,0.36] (0.81)	0.01 [-0.39,0.40] (0.98)	0.10 [-0.41,0.60] (0.70)
11	12+22	1462	695	767	-0.02 [-0.38,0.33] (0.90)	0.14 [-0.32,0.60] (0.55)	-0.17 [-0.70,0.37] (0.53)
12+22	12+22	2995	1491	1504	-0.02 [-0.54,0.07] (0.13)	-0.30 [-0.68,0.31] (0.11)	-0.16 [-0.64,0.31] (0.51)
22	22	174	80	94	**-0.91 [-1.61, -0.2] (0.01)**	-0.82 [-1.75, 0.11] (0.08)	-0.99 [-2.02, 0.05] (0.06)

FABP2 promoter (rs6857641), MTTP Q297H (rs2306985)

One advantage of GWA is the possibility to rapidly find potential gene-gene interaction candidates, which needs more preliminary results in candidate gene approaches. Since male carrier of the FABP2 promoter haplotype B and female MTTP H297Q SNP carrier both displayed reduced BMI in the KORA cohort a combined gene-gene interaction association analysis was carried out [unpublished]. Unlike anticipated, no significant association of the common genotype against one or both rare genotypes was present after gender stratification or in heterozygous individuals. Only unstratified homozygous carrier of both rare polymorphisms exhibited a BMI reducing effect (-0.91 BMI unit, p=0.01) (Table 3). The borderline significance in the gender subgroups (males: -0.82 BMI units, p=0.08; females: -0.99 BMI units, p=0.06) may be caused by too small subgroups (males: n=80; females: n=94). In conclusion, even if strong phenotypes could be confirmed, results from polymorphism specific regulations can not simply be summarised.

For understanding the complexity of the MetS GWAs will not be the spearhead in life science research. Future investigators will use system based approaches, consisting of GWAs and mass spectrometry in combination with computational and statistical tools leading to genomic-metabolomic-networks [190].

A genomic-metabolomic network needs to incorporate environmental factors into data interpretation to enhance result quality. While environment includes numerous variables, which could not completely be accounted for, interaction with nutrition and particularly fat is of special interest for the present candidate genes.

Independent of the total amount, the type of dietary fat became more important during the past few years. The initially recommended <30% of total energy from fat, 2.5% Ω6 and 0.5% Ω3 FA [194] is subdivided into <7% from saturated fat (SFA), minimal trans fat (TFA) and <200mg/d cholesterol. Multiple dietary intervention studies demonstrated beneficial effects of monounsaturated FA (MUFA) and polyunsaturated FA (PUFA) for insulin sensitivity [195]. Interestingly, in animal models MUFA substitution of SFA was not atheroprotective *per se* indicating a more complex FA interaction pattern [196]. The pro-inflammatory potential of Ω6 as well as the anti-inflammatory potential of Ω3 PUFAs is well established [197], nevertheless, the Ω3-ascribed protective effect against T2D was not confirmed in meta analysis [195]. SFA level >15% of daily energy intake are associated with adverse metabolic effects. When provided moderate and balanced with PUFAs SFAs showed lipogenic, amid linkage, bioavailability enhancing, PUFA precursor, cancer prevention and apoptosis initiating characteristics ascribing SFAs new and important cell regulatory functions [198].

Nevertheless, dietary fat intervention studies are numerous with different study settings leading to divergent but often well examined outcomes. Some individuals are relatively insensitive (hypo-responders) to dietary intervention, whereas others (hyper-responders) show enhanced sensitivity [195]. This may partly be due to polymorphisms in fat metabolism genes. Variation in dietary fat-dependent gene regulation was stated earlier for the FABP2 polymorphisms [71-76], while SNP specific nutrigenetic interaction for MTTP shows no associations [199] and for PTGES2 awaits elucidation. In general, FABP2 and MTTP influence gene regulation via FA regulated TFs which increase transcription of FA transport/metabolism and gluconeogenesis genes but also reduce FA oxidation and carbohydrate processing genes [18, 167]. While PUFAs regulate transcription via PPARs and SREBP, SFAs strongly elevate PGC1β, interact with HNF4, NF-κB, C/EBP and PPARα

[198]. PUFA (Ω6) mediated gene regulation is further influenced by PTGES2 which is relevant for AA processing [200].
Depending on the variety of dietary composition and its influence on gene regulation there are still numerous possibilities to perform candidate gene and GWA analysis.

In summary, association analysis in the KORA cohort with more than 8000 participants revealed association between BMI and polymorphisms of the FABP2 promoter and the MTTP and PTGES2 genes. The MTTP SNP H297Q and related haplotypes are also associated with waist circumference and total cholesterol. Functional analysis exhibited, that the FABP2 promoter haplotype B is 2-fold more inducible by PPARγ/RXRα than haplotype A. This difference is caused by polymorphism c.-471G>A. In addition, OCT1 was identified as a TF regulating the FABP2 promoter. In conclusion, variants of the FABP2, MTTP and PTGES2 genes, which are involved in fat metabolism, contribute to the development of the MetS.

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Research Article

Association between functional FABP2 promoter haplotypes and body mass index: Analyses of 8072 participants of the KORA cohort study

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Studies in relatively small cohorts provide preliminary evidence that functional fatty acid binding protein 2 (FABP2) promoter haplotypes are associated with type 2 diabetes and BMI. Here, we studied the influence of the haplotypes on BMI by using 8072 male and female participants of the Kooperative Gesundheitsforschung in der Region Augsburg (KORA) cohort. By linear regression analysis, we found in males a reduction of −0.39 BMI units (95% CI: −0.73, −0.05, $p = 0.024$) in homozygous FABP2 promoter haplotype B carriers. Carriers of haplotype B showed a significant decrease in BMI of −0.19 BMI units (95% CI: −0.35, −0.02, $p = 0.027$). In accordance, a significant reduction in BMI of the minor haplotype carriers in the BMI point categories of 25–30 (BMI units: −0.10, 95% CI: −0.18, −0.01, $p = 0.03$) and <30 (BMI units: −0.37, 95% CI: −0.67, −0.07, $p = 0.02$) were found. In summary, the minor FABP2 promoter haplotype B contributes to a reduced BMI in men. This provides evidence that functional FABP2 contributes to multifactorialy regulated body weight.

Keywords: BMI / FABP2 gene / KORA / Promoter polymorphism

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1 Introduction

The human fatty acid binding protein 2 (FABP2) gene (*FABP2*, NM_000134.2) is exclusively expressed in the intestine during development and cell differentiation in absorptive cells of the upper villus region [1–3]. Although the specific function of FABP2 is still not fully elucidated, the protein is proposed to be involved in fat absorption by binding and intracellular transport of newly absorbed non-esterified long-chain free fatty acids, which are finally assembled and secreted in triglyceride-rich chylomicrons [4, 5]. FABP2 also mediates fatty acid signalling by transferring fatty acids to the nucleus as ligands for transcription factors, such as peroxisome proliferator-activated receptors (PPARs) [6, 7] and seems to be involved in regulation of cell proliferation and differentiation [8, 9]. More recently, an antioxidant function was shown [10, 11].

Based on a huge number of association studies in different human populations, *FABP2* is described as a candidate gene for the metabolic syndrome [12–16]. Sequencing studies [13, 14, 16] revealed that the 5′ upstream promoter region of *FABP2* contains insertion/deletion sites c.–80_-79insT (rs5861422), c.–136_-132delAGTAG (rs5861423) and c.–168_-166delAAGinsT (rs1973598) as well as c.–260G > A (rs6857641), c.–471G > A (rs2282688) and c.–778G > T (rs10034579) single nucleotide polymorphisms (SNPs). For explanation of sequence variation nomenclature refer to den Dunnen and Antonarakis, 2000 [17]. These six variants are in complete linkage disequilibrium resulting in only two haplotypes A > B. As demonstrated by us [16] and others [14] the rare *FABP2* promoter haplotype B, with an allele frequency in Caucasians of 44% [16], showed almost 2–3-fold lower transcriptional activity than haplotype A in Caco2 cells. Recently, we showed that this different activity is due to decreased binding affinity of GATA-5 and -6 to the c.–80_-79insT polymorphism [18]. The *FABP2* promoter haplotypes were associated with postpran-

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Abbreviations: FABP2, fatty acid binding protein 2; **KORA,** Kooperative Gesundheitsforschung in der Region Augsburg; **MONICA,** multinational MONItoring of trends and determinants in Cardiovascular disease; **PPAR,** peroxisome proliferator-activated receptor

dial triglyceride levels [15], type 2 diabetes [16] and BMI [13]. Since, these studies were done in relatively small cohorts composed of 500–1000 participants and functional intervention studies are still lacking, the associations remain disputable. In the present study, we analysed the data of a large (n = 8072) population-based survey from 1994 to 2001 of the Kooperative Gesundheitsforschung in der Region Augsburg (KORA) study cohort regarding the association between *FABP2* promoter haplotypes and BMI.

2 Materials and methods

2.1 Study population

In the Southern German region of Augsburg including the city of Augsburg and the two surrounding counties, population-based surveys of the 25–74 years old population in groups of age range of 5 years were implemented in 1984 as part of the WHO multinational MONItoring of trends and determinants in CArdiovascular disease (MONICA) project and continued since, 1996 within the KORA platform. The aims of the surveys were to describe the prevalence of common diseases and their risk factors in a representative sample of the adult general population. The current study included the survey of the years 1994–1995 (MONICA S3) including 4856 participants and the survey of the years 1999–2001 (KORA S4) including 4261 participants yielding 9117 recruited participants. The study population of S3 and S4 consisted of all German residents of the Augsburg region who were born between 1920 and 1975 identified through the public record office. More than 99.5% of the participants were Caucasian. The high standards of the WHO MONICA project applied to both surveys. All study participants underwent a standardized interview, a physical examination and blood withdrawal by trained staff and signed a consent form of the Bavarian Ethics committee and the Ethics committee of the University of Munich. For determination of body weight and height, participants were asked to remove shoes and heavy clothing. The weight measurements were done with a calibrated body weight scale (SECA 709) and were carried out with an accuracy of 0.1 kg. The body height was read to the nearest 0.5 cm on a body height scale. BMI (kg/m^2) was calculated as weight in kilograms divided by height in square meters. The pooled data analysis included 8072 individuals with complete information on age, sex, BMI and genotypes. The range of BMI was 14–57 kg/m^2 and the gender distribution was equal. There was no overlap between the two surveys by design. Detail characteristics of the study cohorts were described elsewhere [19, 20].

2.2 Genotyping

The 5′ upstream promoter region of *FABP2* containing insertion/deletion sites c.–80_-79insT (rs5861422), c.–136_-132delAGTAG (rs5861423) and c.–168_-166delAAGinsT (rs1973598) as well as c.–260G > A (rs6857641), c.–471G > A (rs2282688) and c.–778G > T (rs10034579) is in complete linkage disequilibrium resulting in only two haplotypes A > B. *FABP2* promoter haplotypes were genotyped based on htSNP c.–260G > A (rs6857641) using a MALDI TOF MS system (Sequenom, Mass EXTEND, San Diego, USA), with the 5′ Capture Primer ACGTTGGATGAACAATCTTCAGACGGCATG and the 3′ Capture Primer ACGTTGGATGTGAGAAGCATACCTATTCTG.

2.3 Statistical methods

We used a linear regression model with BMI as continuous outcome for both studies combined; the unit change in BMI by the DNA variants was estimated. Haplotype analysis was carried out with the statistical software R (V. 2.3.1. including haplo.stats package) using the haplo.glm procedure. This procedure performs an iterative two-step estimation-maximization (EM)-algorithm, with the posterior probabilities of pairs of haplotypes *per* subject used as weights to update the regression coefficients, and the regression coefficients used to update the posterior probabilities. Haplotypes were included into the regression model all at once except the most common haplotype. Using the expected number of copies of haplotype implies an additive model for each haplotype. Statistics were gender-stratified because sex-specific associations of FABP2 polymorphisms were described [21, 22].

3 Results and discussion

As shown in Table 1, in the whole study population as well as in female subjects no associations between *FABP2* promoter haplotypes and BMI were found. In male subjects, we observed a significant reduction in BMI of –0.39 BMI units (95% CI: –0.73, –0.05, p = 0.024) for the homozygous *FABP2* promoter haplotype B compared with homozygous haplotype A (wild type). Carriers of haplotype B (A/B + B/B) showed a significant decrease in BMI of –0.19 BMI units (95% CI: –0.35, –0.02, p = 0.027). These findings were congruent with a significant reduction in BMI of the minor haplotype carriers (A/B + B/B) in the BMI point categories of 25–30 (BMI units: –0.10, 95% CI: –0.18, –0.01, p = 0.03) and <30 (BMI units: –0.37, 95% CI: –0.67, –0.07, p = 0.02).

The presented analysis of 8072 participants confirmed and extended previously data in humans that the rare *FABP2* promoter haplotype B has a protective effect on metabolic traits like type 2 diabetes [16]. Though, previously an increase in BMI was found in female non-Hispanics from Colorado (USA) [13] and no decrease in male whites similar to our results. These differences may partly be due to different diet composition, especially fatty acids,

Table 1. Frequencies of FABP2 promoter genotypes (AA, AB, BB) and their associations with BMI as continuous variable *via* linear regression

	Frequencies			Unit change in BMI in kg/m^2 [95% confidence interval] (*p*-values)		
	All	Men	Women	All	Men	Women
By genotype						
All	8072	4002	4070			
AA	2695	1293	1402			
AB	3974	1997	1977	−0.047 [−0.26, 0.16] (0.66)	−0.140 [−0.4, 0.12] (0.29)	0.048 [−0.28, 0.38] (0.78)
BB	1403	712	691	−0.206 [−0.49, 0.07] (0.15)	−0.392 [−0.73, −0.05] (0.024)	−0.004 [−0.44, 0.43] (0.99)
B[a)]	5377	2709	2668	−0.094 [−0.23, 0.04] (0.18)	−0.188 [−0.35, −0.02] (0.027)	0.007 [-0.21, 0.22] (0.95)
By WHO BMI cat.[a)]						
18.5–25	2729	1044	1685	−0.041 [−0.12, 0.04] (0.33)	0,011 [−0.11, 0.13] (0.86)	−0.072 [−0.18, 0.04] (0.21)
25–30	3507	2105	1402	−0.067 [−0.13, 0] (0.049)	−0.098 [−0.18, −0.01] (0.025)	−0.021 [−0.13, 0.09] (0.71)
30–35	1355	695	660	0.037 [−0.07, 0.14] (0.51)	0.056 [−0.09, 0.2] (0.46)	0.017 [−0.14, 0.17] (0.83)
35–40	334	116	218	0.029 [−0.19, 0.24] (0.79)	0.111 [−0.28, 0.5] (0.57)	−0.011 [−0.27, 0.25] (0.93)
By obesity status[a)]						
<30	6282	3159	3123	−0.16 [−0.39, 0.07] (0.17)	−0.37 [−0.67, −0.07] (0.017)	0.01 [−0.33, 0.36] (0.94)
330	1790	843	947	−0.06 [−0.15, 0.03] (0.17)	−0.10 [−0.22, 0.02] (0.10)	−0.02 [−0.16, 0.12] (0.76)

Results are the β estimate (linear regression) with 95% confidence interval and *p*-values. All calculations were adjusted for age and sex. The main results are in bold.

a) Additive model.

in the two cohorts. Additional, neither this nor the study from Damcott *et al*. 2003 [13] were adjusted for medicament treatment of participants, which might have an impact on fatty acid metabolism and lead to divergent results.

Differences in associations of the *FABP2* promoter haplotypes with metabolic traits may result from strong but not total linkage disequilibrium of the promoter haplotype B with the Thr allele of the *FABP2* Ala54Thr polymorphism with an LD = 0.9 [13]. Associations with the Thr allele and metabolic traits showed controversial results. The Thr allele was associated with increased fasting lipid metabolism, intra-abdominal fat, C reactive protein, IL6, lipoprotein a, fasting insulin concentration, fasting fatty acid oxidation and decreased insulin sensitivity, reduced glucose uptake and obesity [23–29]. Further studies showed no influence of the Ala54Thr polymorphism on fasting and postprandial insulin, glucose or triglyceride levels, fat distribution and weight loss [30–35]. Other studies found associations with reduced subcutaneous adipose tissue, systolic blood pressure and glucose levels [31, 36]. These studies were carried out in populations with different ethnic, phenotypic and lifestyle backgrounds. This contributes in addition to putative changes in LD between the promoter and exon polymorphism in these different races to diverse associations.

Further, our data revealed a reduced BMI for nonobese but not for obese males carrying haplotype B. Hence, the *FAPB2* promoter haplotype B may bear prevention against overweight as long as obesity has not been occurred.

At a first glance, the observed sexual dimorphism shows inconsistencies with the phenotype of *FABP2* knock out mice [37]. Male mice lacking FABP2 have higher body weight than wild type animals [37]. Here, a reduced BMI is associated with the *FABP2* promoter haplotype B which showed lower basal activity than haplotype A in cell culture experiments [13, 14, 16, 18, 38]. Assuming a functional connection of *FABP2* gene expression and BMI we tempt to speculate that the *FABP2* promoter haplotype B is *in vivo* more active in males than in females. This is supported by reports demonstrating higher activity of PPARs in male animals compared to females [39, 40] and higher responsiveness of *FABP2* promoter haplotype B than A to PPARg/RXRa as recently stated by us and others [41, 42]. The physiological mechanism by which the *FABP2* promoter influences BMI remains obscure. A possible pathway is the complex and still incomplete understood interaction between diabetes type 2 and obesity [27, 43]. FABP2 is not solely expressed in enterocytes but also in GLP expressing intestinal epithelial L-cells [44]. The anti-diabetic hormones GLP-1 and GLP-2 are expressed after fat ingestion and could influence insulin sensitivity and *via* glucose metabolism the triglyceride metabolism in peripheral cells. Of course our hypothesis has to be tested by comparing the FABP2 expression levels from human males and females.

4 Concluding remarks

We obtained evidence in a population-based sample comprising 8072 participants that the minor *FABP2* promoter haplotype B contribute to a reduced body weight in men.

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Analysis of the transcriptional regulation of the *FABP2* promoter haplotypes by PPARγ/RXRα and Oct-1

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ABSTRACT

Variants of the human intestinal fatty acid binding protein 2 gene (FABP2) are associated with traits of the metabolic syndrome. Relevant FABP2 promoter polymorphisms c.-80_-79insT, c.-136_-132delAGTAG, c.-168_-166delAAGinsT, c.-260G>A, c.-471G>A, and c.-778G>T result in two haplotypes A and B. Activation of haplotypes by rosiglitazone stimulated PPARγ/RXRα leads to 2-fold higher activity of haplotype B than A. As shown by chimeric FABP2 promoter constructs, the higher responsiveness of FABP2 haplotype B is mainly but not solely determined by polymorphism c.-471G>A. As shown by EMSA and promoter–reporter assays, Oct-1 interacts with the −471 region of FABP2 promoters, induces the activities of both FABP2 promoter haplotypes and abolishes the different activities of haplotypes induced by rosiglitazone activated PPARγ/RXRα. In conclusion, our findings suggest a functional role of PPARγ/RXRα and Oct-1 in the regulation of the FABP2 gene.

1. Introduction

The human fatty acid binding protein 2 gene (FABP2, GenBank: NM_000134.2) is located on chromosome 4q28-q31, contains four exons and codes for a 15.1-kDa protein comprising 132 amino acid residues [1]. It is expressed in the intestine with highest cellular concentrations in the jejunum [2,3]. FABP2 is involved in intestinal fat absorption [4–6]. A huge number of studies in different human populations revealed associations between the minor allele of the FABP2 Ala54Thr polymorphism and risk parameters of insulin resistance [7]. Functional studies showed increased binding affinity of the variant protein to long-chain fatty acids [8]. More recently [9–11], single nucleotide polymorphisms and insertion/deletion sites c.-80_-79insT, c.-136_-132delAGTAG, c.-168_-166delAAGinsT and SNPs -260G>A, c.-471G>A and c.-778G>T were identified in the 5¢ upstream promoter region of FABP2. These polymorphisms are in complete linkage disequilibrium resulting in only two haplotypes A>B. FABP2 promoter haplotypes were associated with postprandial triglyceride levels [12], BMI [9] and type 2 diabetes [11]. In accordance with the association studies, male FABP2 knock-out mice showed key symptoms of the metabolic syndrome [13,14]. Therefore, the expression level of FABP2 seems to be critical for insulin sensitivity and triglyceride metabolism. Thus, the transcriptional regulation of the FABP2 gene is of special interest. In the present study we focus on the regulation of FABP2 promoter haplotypes by PPARγ/RXRα and Oct-1.

2. Materials and methods

2.1. Cell culture

HeLa and CaCo2 cells were purchased from German National Resource Centre for Biological Material (DSMZ, Braunschweig, Germany). Cells were maintained in MEM (Invitrogen, Carlsbad, CA, USA), supplemented with 10% (HeLa) or 20% (CaCo2) fetal calf serum (Invitrogen) and 1 mM non-essential amino acids (PAA, Cölbe, Germany) in a humidified incubator at 37 °C under an atmosphere of 5% CO_2. Cells were passaged at pre-confluent densities by use of 0.05% trypsin/5 mM EDTA (Biochrom AG, Berlin, Germany) solution every 2–3 days.

2.2. FABP2 promoter luciferase constructs

The dual luciferase system was used (Promega, Madison, WI, USA). Cloning procedures were performed using Gateway Technology (Invitrogen), described previously [11,15].

2.3. Chimera constructs

Mutations in haplotype A (pGL4.10[luc2]–FABP2(A)) and haplotype B (pGL4.10[luc2]–FABP2(B)) containing promoter constructs were introduced to generate chimeras with single polymorphisms of haplotype B in the background of haplotype A (A-xB) and vice versa (B-xA). Single polymorphism exchange chimera constructs are shortly named as follows: for exchange of polymorphism c.-80_-79insT of haplotype A (rs5861422): A-80B; for c.-136_-132delAGTAG

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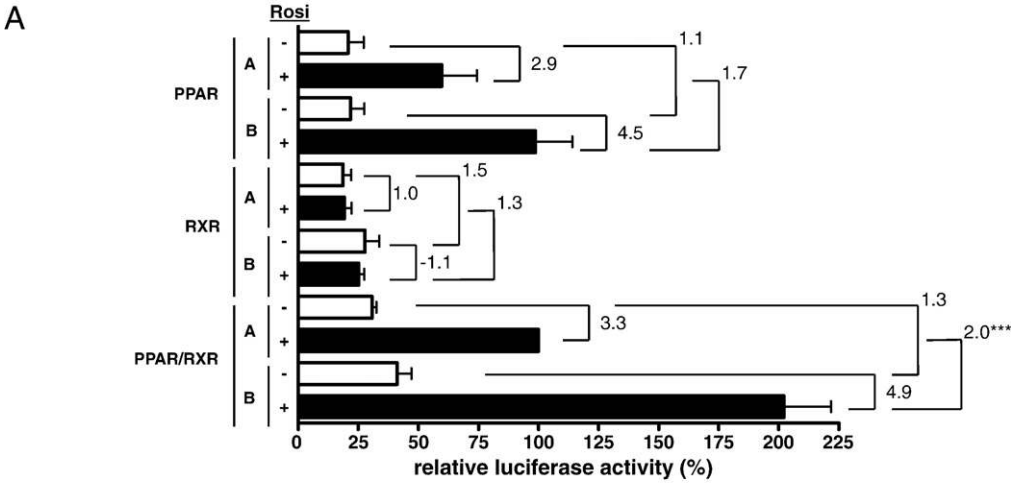
(rs5861423): A-136B; for c.-168_-166delAAGinsT (rs1973598): A-168B, c.-260G>A (rs6857641): A-260B c.-471G>A (rs2282688): A-471B; c.-778G>T (rs10034579): A-778B and vice versa. Mutation of a putative PPARγ binding site at −623/−607 upstream of the translation start site was introduced into the haplotype A (pGL4.10 [luc2]–FABP2(A)) and haplotype B (pGL4.10[luc2]–FABP2(B)) containing promoter by exchange of a 4 base core region from CTAT to AGCG named Amut and Bmut, respectively. Introduction of mutations was performed using Quick Change in vitro mutagenesis Kit (Stratagene, La Jolla, CA, USA). All primers were purchased from MWG Biotech AG (Ebersberg, Germany). Sequences of primers are available on request. Verification of constructs was performed by sequencing.

2.4. Expression plasmids

The cDNAs of PPARγ2 and RXRα were kindly provided by Dr. T. Weitzel (University Medical Centre Hamburg-Eppendorf, Germany). Cloning procedures were performed using Gateway Technology (Invitrogen) with the pcDNA-Dest40 destination vector as described previously [11,15,16]. The expression plasmids pcDNA3HA-Oct-1 and its empty control vector were a gift from H. Singh (University Chicago, IL, USA).

2.5. Transient transfections and reporter assays

Transient transfections were performed with FuGene6 (Roche, Basel, Switzerland) according to the manufacturers instructions. 4×10^3 HeLa cells were plated in 96-well plates. Cells were transfected with 30 ng (Figs. 1–2) or 25 ng (Fig. 3) pGL4.10[luc2]–FABP2-Promoter constructs or pGL4.10[luc2] as negative control and 3 ng pGL4.74 [hRluc/TK] vector encoding *Renilla* luciferase as internal control. For co-transfection with PPARγ/RXRα 33 ng (Figs. 1–2) or 25 ng (Fig. 3) Dest40-PPARγ and RXRα expression vectors and 25 ng Dest40 empty control vector (Fig. 3) were used. For co-transfection with Oct-1 25 ng pcDNA3HA-Oct-1 and 75 ng empty pcDNA3HA vector were added. Co-transfection with PPARγ/RXRα/Oct-1 was carried out with 25 ng of each plasmid vector. The PPARγ/RXRα complex was activated 24 h



B

	Rosi	Fold-change to A -	Fold-change to A +
A	-	0	-2.7
	+	2.7	0
A-80B	-	1.2	-
	+	-	1.1
A-136B	-	1.1	-
	+	-	-1.1
A-168B	-	1.1	-
	+	-	-1.1
A-260B	-	1.1	-
	+	-	**-1.5*** **
A-471B	-	-1.3	-
	+	-	**1.4** **
A-778B	-	1.1	-
	+	-	1.0
B	-	1.1	-
	+	-	**2.0*** **
B-471A	-	-1.4	-
	+	-	1.1

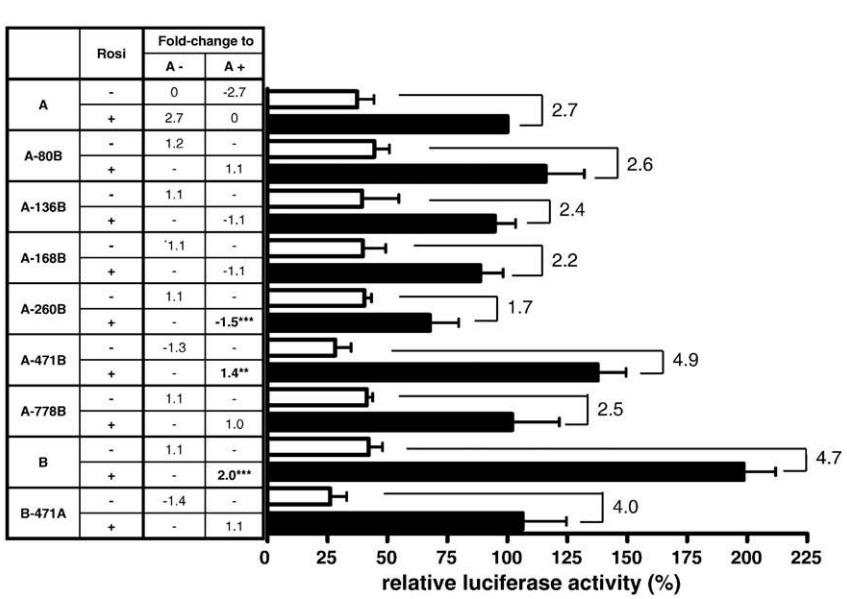


Fig. 1. Reporter activities of FABP2 promoter haplotypes and from it derived chimeras in HeLa cells were assessed 48 h after co-transfection with PPARg and/or RXRα and 24 h after activation with rosiglitazone (Rosi) (+) or DMSO control (−) using dual luciferase assay. The *Firefly* luciferase activities were normalized to *Renilla* luciferase activities and are given as relative luciferase activities in % of FABP2 promoter haplotype A. Each experiment was performed in triplicate for each sample. The results are expressed as mean±SEM for three independent experiments. ***$p<0.001$, **$p<0.01$, *$p<0.05$, as compared with haplotype A activity in all figures. (A) Luciferase activities of FABP2 promoter haplotypes (A, B) co-transfected with PPARγ, RXRα or PPARγ/RXRα are shown. (B) Alleles of each polymorphism in the FABP2 promoter haplotypes A>B are given. The positions of polymorphisms c.-80_-79insT (rs5861422), c.-136_-132delAGAGT (rs5861423), c.-168_-166delAAGinsT (rs1973598), c.-260G>A (rs6857641), c.-471G>A (rs2282688), and c.-778G>T (rs10034579) are named as -80, -136, -168, -260, -471, and -778, respectively.

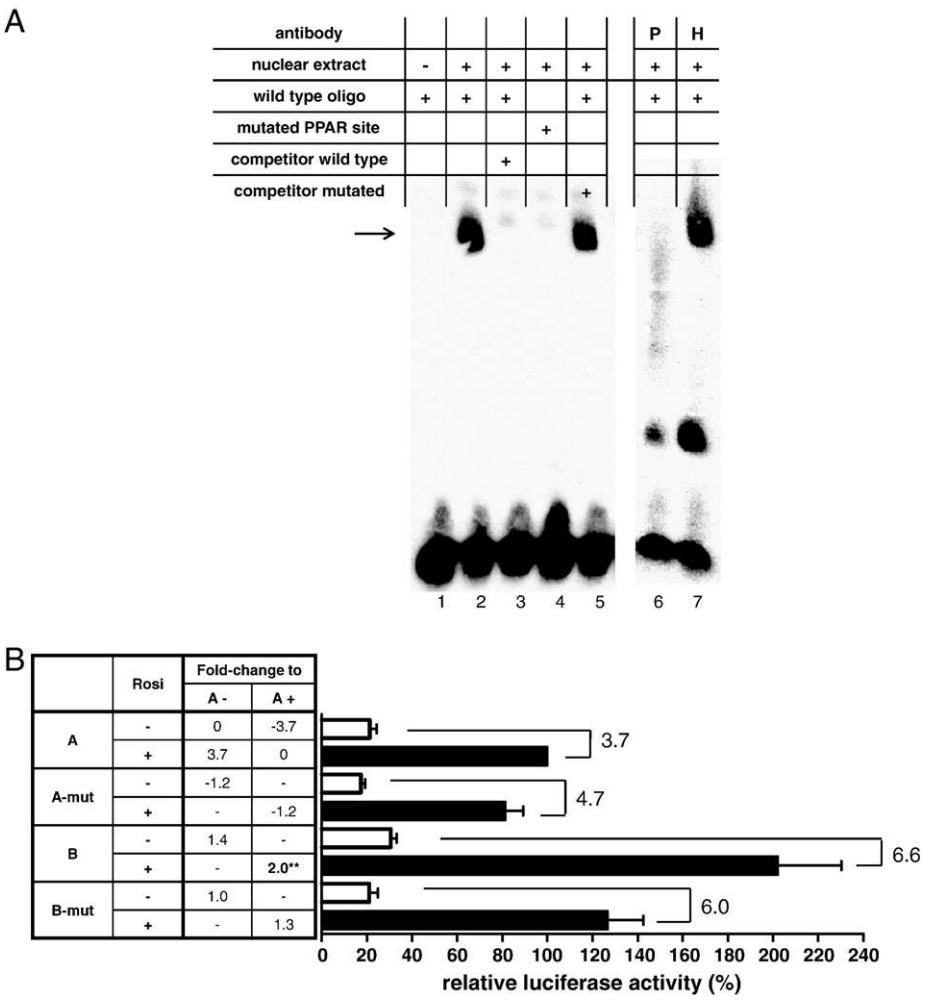


Fig. 2. (A) Differential interaction of PPARγ with the FABP2 promoter region −623/−607 was analyzed with EMSA. Nuclear extract isolated from HeLa cells transfected with PPARγ/RXRα were used for each binding reaction (no extract was added in lane 1) with biotinylated wild type oligos (lanes 1–3, 5–7) or mutated PPARγ binding site oligo (lane 4). A specific shift was diminished by 100-fold excess of unlabeled oligo (lane 3) but not by unlabeled mutated PPARγ binding site oligo (lane 5), indicated with an arrow. Super shift assays were carried out with antibodies against PPARγ (P, lane 6) and HNF4α (H, lane 7). (B) Reporter activities of FABP2 promoter haplotypes (A, B) and from it derived chimeras with a mutated PPARγ core binding site at position −619/−616 were assessed in HeLa cells 48 h after co-transfection with PPARγ/RXRα and 24 h after activation with rosiglitazone (Rosi) (+) or DMSO control (−) using dual luciferase assay.

after co-transfection with medium containing 1% NEAA, 1% BSA and 2 μM rosiglitazone or DMSO as negative control. Luciferase activities were measured 48 h after transfection by Dual-Luciferase® Reporter Assay System (Promega). Relative luciferase activities of negative control pGl4.10[luc2] were determined for each condition and were subtracted from corresponding activities for pGL4.10[luc2]–FABP2-Promoter constructs. Each experiment was repeated at least three times, and each sample was studied in triplicate.

2.6. Electrophoretic mobility shift assay (EMSA)

EMSA was done as previously described [15,17]. The oligonucleotide sequences for position −629/−602 are 5′-GCTCAGTGGCCTATCACCATTCAGGATC-3′ for the wild type and 5′-GCTCAGTGGCAGCGCACCATTCAGGATC-3′ for the mutated PPARγ binding site oligo, and for position −488/−447 are 5′-GAAAACGAATCTTATTAACTTTAGCTTTTCAACTGTATTTC-3′ for the wild type and 5′-GAAAACGAATCTTATTAACGGGCGCTTTTCAACTGTATTTC-3′ for the mutated Oct-1 binding site oligo, respectively. The mutated core binding sites are underlined; the base −471 is italicized. Antibodies again PPARγ2 (sc-22020) and HNF4α (sc-6547x) were purchased from Santa Cruz (Heidelberg, Germany) and for Oct-1 self-made by C. Brunner (University Ulm, Germany) and added to a final concentration of 1 μg (PPARγ) and 2 μg (Oct-1 and HNF4).

2.7. Real time RT-PCR

For RNA analysis 5.2×10^4 CaCo2 cells were plated in 6-well plates and transfected with 4 μg pcDNA3HA-Oct-1 or solely Fugene 6 as control. Total RNA was extracted 48 h and 120 h after confluent growth with the RNeasy Kit (Qiagen, Hilden, Germany). CDNA synthesis of 1 μg total RNA was done with Omniscript RT (Qiagen). Real time PCR was performed with a 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) using QuantiTect SYBR Green PCR Kit (Qiagen). Primer were designed with the Primer3 online tool [18] and purchased from MWG Biotech AG. Sequences are available upon request. To determine the relationship between cycle number (C_T) and mRNA levels the $\Delta\Delta C_T$ method was conducted using 18S as control gene. All procedures were carried out according to manufactures instructions.

2.8. Statistical analyses

Statistical analyses were performed with GraphPad Prism 4 (GraphPad Software, Inc., San Diego, CA) using ANOVA and unpaired *t*-tests. The differences between haplotype A and B activities were tested with two-tailed *t*-tests. For comparison of chimera activities with haplotype A we used one-tailed *t*-tests because we hypothesized that activities of the chimera promoter constructs have to be above

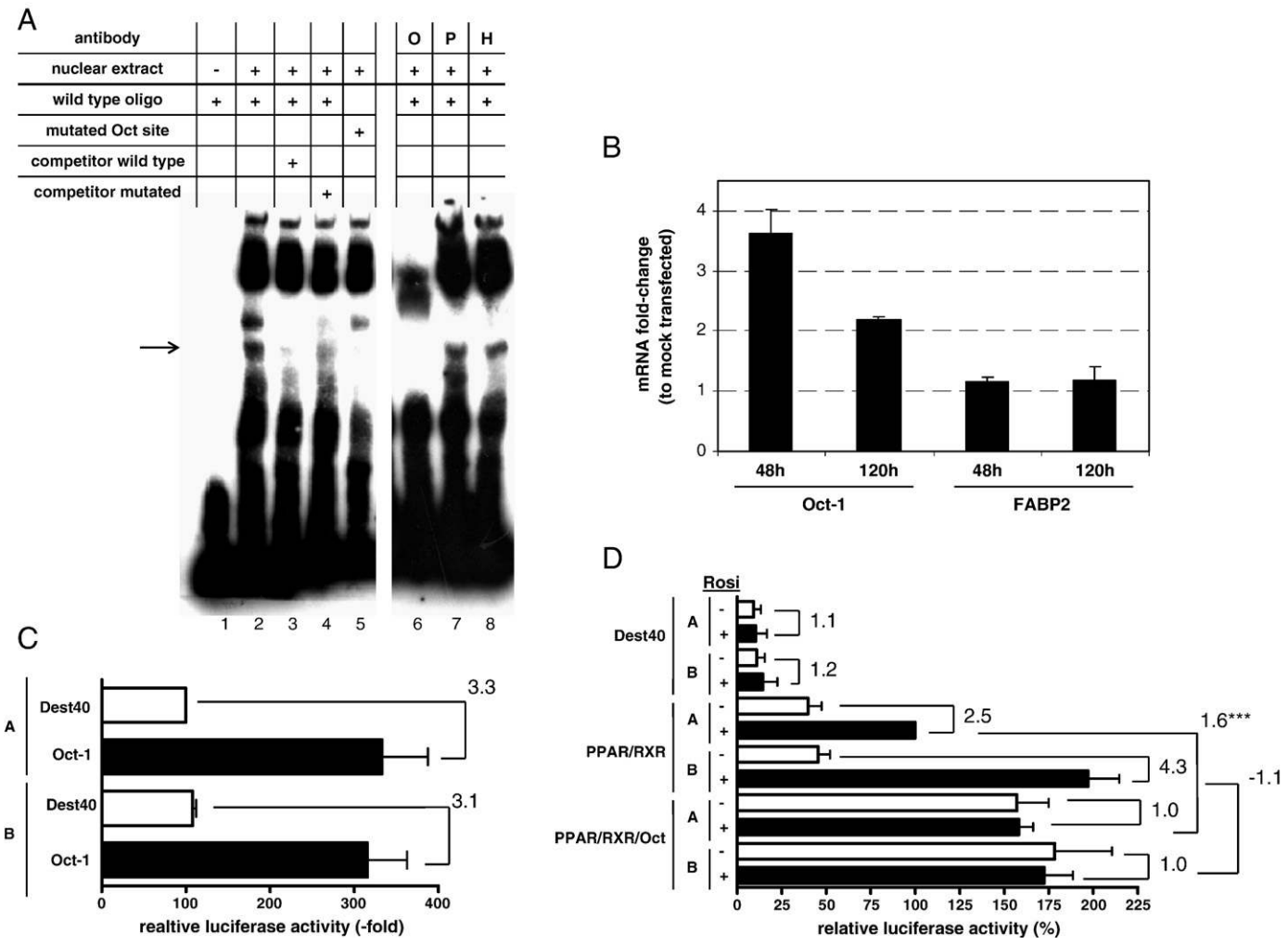


Fig. 3. (A) Differential interaction of Oct-1 with FABP2 promoter region containing the allele A of polymorphisms c.-471G>A (rs2282688) was analyzed with EMSA. Nuclear extract isolated from HeLa cells was used for each binding reaction (no extract was added in lane 1) with biotinylated haplotype A oligonucleotides (lanes 1–4, 6–8) or biotinylated mutated Oct-1 binding site oligonucleotide (lane 5). A specific shift was diminished by 100-fold excess of unlabeled haplotype A oligo (lane 3) but not by unlabeled mutated Oct-1 binding site oligo (lane 4), indicated with an arrow. Super shift assay was carried out with an antibody against Oct-1 (O, lane 6) and control antibodies again PPARγ (P, lane 7 and HNF4α (H, lane 8). (B) Gene expression of Oct-1 and FABP2 in Caco2 cells was assessed 72 h after co-transfection and 48 h or 120 h after post-confluence with Oct-1 using SYBR green RT-PCR. Results are measured with the $\Delta\Delta C_T$ method and 18S as control gene and are expressed as fold-change against Fugene 6 transfection. Each experiment was performed in triplicate for each sample. The results are expressed as mean ± SEM for two independent experiments. (C). Reporter activities of FABP2 promoter haplotypes (A, B) in HeLa cells were assessed 48 h after co-transfection with empty control vector Dest40 or Oct-1 using dual luciferase assay. The *Firefly* luciferase activities were normalized to *Renilla* luciferase activities and are given as relative luciferase activities in % of Dest40 transfected FABP2 promoter haplotype A. (D) Reporter activities of FABP2 promoter haplotypes (A, B) in HeLa cells were assessed 48 h after co-transfection with empty control vector Dest40, PPARγ/RXRα, or PPARγ/RXRα/Oct-1 and 24 h after activation with rosiglitazone (Rosi) (+) or DMSO control (−) using dual luciferase assay.

promoter activities of haplotype A. All values were expressed as means ± SEM. Significant differences were considered for p values less than 0.05.

3. Results

3.1. The polymorphism c.-471G>A of FABP2 promoter haplotypes is involved in determining different activities of FABP2 promoter haplotypes induced by rosiglitazone activated PPARγ/RXRα

As published recently [19], activation of FABP2 promoter haplotypes by rosiglitazone stimulated PPARγ/RXRα leads to higher activity of haplotype B than A. As shown in Fig. 1A, the rosiglitazone stimulated PPARγ/RXRα induced absolute activity (AA) of haplotype B is 2.0-fold higher than haplotype A. The induction ratio (IR) between unstimulated and rosiglitazone stimulated PPARγ/RXRα induced activity is 3.3 for haplotype A and 4.9 for haplotype B. To identify the causal polymorphism(s) for this haplotype difference, we sequentially introduced alleles of FABP2 haplotype B into A (A-xB) and compared the rosiglitazone stimulated PPARγ/RXRα induced activities of resulting FABP2 promoter chimeras with those of haplotype A and B. As shown in Fig. 1B, AA of FABP2 promoter chimeras A-80B (1.1-fold compared to A), A-136B (-1.1-fold), A-168B (-1.1.-fold), and A-778B (1.1-fold) is similar to haplotype A (1.0-fold). AA of chimera A-471B (1.4-fold) is between haplotype B (2-fold) and A (1.0-fold). The opposite chimeras B-471A (1.1-fold) showed similar AA than haplotype A. Comparison of IRs revealed that chimera A-471B (4.9-fold) exhibits similar IR than haplotype B (4.7-fold). The IR of the opposite chimeras B-471A (4.0-fold) is more similar to haplotype B (4.7-fold) than A (2.7-fold). Thus, we reasoned that the polymorphism c.-471G>A of FABP2 promoter haplotypes is involved in determining rosiglitazone stimulated PPARγ/RXRα induced activities of haplotype A and B.

3.2. Identification of a PPARγ binding site at position −623/−607 of the FABP2 promoter

A TRANSFAC Professional database search revealed a putative PPARγ binding site at position −623/−607 of the FABP2 promoter. This

site was tested for PPARγ binding using EMSA. As shown in Fig. 2A, with a −629/−602 probe we obtained a shift (arrow, lane 2) when using nuclear extract from HeLa cells transfected with PPARγ/RXRα. 100-fold molar excess of the unlabeled probe (lane 3) but not a mutated probe (lane 5) abolished the shift. A labeled probe with a mutated PPARγ core binding site did not display a shift (lane 4). A super shift assay with an antibody against PPARγ (lane 6) but not HNF4α (lane 7) abrogated the shift. To explore the functional importance of the −623/−607 region for FABP2 promoter haplotype difference, promoter–reporter analysis with haplotype mutants was done. As shown in Fig. 2B, incorporation of a 4 bp mutation into the core sequence of PPARγ-consensus at position −619/−616 leads to a reduction of the AA of FABP2 promoter haplotypes by a factor of −1.2 (haplotype A) and −1.6 (B), respectively. Mutation of the putative PPARγ binding site shows a small decrease or increase in IR for haplotype A (from 3.7-fold to 4.7-fold) and B (6.6-fold to 6.0-fold). Taken together, the identified PPARγ binding site at positions −623/−607 of the FABP2 promoter is functional but its influence on the rosiglitazone stimulated PPARγ/RXRα induced activity of *FABP2* promoter haplotypes is limited.

3.3. Oct-1 binds to a FABP2 promoter region containing the c.471G>A polymorphism and abolishes the different activities of FABP2 promoter haplotypes induced by rosiglitazone activated PPARγ/RXRα

A putative Oct-1 binding site in the FABP2 promoter region containing the c.471G>A polymorphism was found by TRANSFAC Professional database search. To see if the −471 region shows binding of the ubiquitous expressed Oct-1 [20], electro mobility shift and super shift assays were performed with nuclear protein extracts from HeLa cells. As shown in Fig. 3A, with a FABP2 promoter probe containing the −471 region we observed a specific signal (lane 2, arrow), which could be displaced with the unlabeled probe (lane 3). This total displacement was not observed by a competitor with a mutation in the Oct-1 core binding site at positions −470/−467 (lane 4). Only unspecific binding was generated by a labeled probe with the mutated Oct-1 binding site (lane 5). A super shift assay with an antibody against Oct-1 (lane 6), but not PPARγ (lane 7) and HNF4α (lane 8), abolished the specific shift completely. Differences in binding affinity to −471G and −471A probes could not be detected by a gradually increasing excess of unlabeled probes (data not shown). Next, we determined the influence of Oct-1 on FABP2 mRNA expression in intestinal CaCo2 cells. As shown in Fig. 3B, a 2–3.5-fold over-expression of Oct-1 has no influence on mRNA steady level of FABP2. In order to reveal the effect of Oct-1 on the transcriptional activity of FABP2 promoter haplotypes we performed promoter–reporter analysis. As shown in Fig. 3C, Oct-1 causes similar activation of FABP2 promoter haplotype A and B. In the presence of PPARγ/RXRα, Oct-1 induces the basal activity of FABP2 promoter haplotype A and B to the same level (Fig 3D). These basal activities could not be induced by rosiglitazone. AA of haplotype A was significantly enhanced (1.6-fold) by Oct-1, whereas AA of haplotype B was not altered (fold-change −1.1). Thus, Oct-1 abolishes the different activities in FABP2 promoter haplotypes induced by rosiglitazone activated PPARγ/RXRα.

4. Discussion

The FABP2 promoter haplotypes are associated with traits of the metabolic syndrome in candidate gene approaches [9,11,12]. Though, in genome-wide association studies the FABP2 locus was linked with physical fitness in DZ female twins [21] but not with type 2 diabetes and related traits [22–24]. As shown previously by us [11] and others [9,10], the basal transcription of the rare FABP2 promoter haplotype B is about 2–3-fold lower than the common haplotype A in FABP2 expressing post-confluent Caco2 cells. Recently, we showed that this different activity is due to decreased binding affinity of GATA factors to the c.-80_-79insT polymorphism [17]. Interestingly, in FABP2 non-expressing HeLa cells activation of FABP2 promoters by PPARγ/RXRα lead to higher activity of haplotype B in comparison to A [19]. In the present study, this higher responsiveness of FABP2 promoter haplotype B was investigated in more detail. At first glance, the rosiglitazone dependent PPARγ/RXRα induced different activities of FABP2 promoter haplotypes seem not to be important *in vivo* since FABP2 is expressed in the small intestine [2] were PPARα and PPARδ are predominantly expressed [25]. Whereas, the higher activating potential of FABP2 promoter haplotype B might result in an ectopic expression of FABP2 in PPARγ expressing tissues of haplotype B carriers. This might explain the FABP2 promoter association with diabetes type 2 [11], since PPARγ is expressed in pancreatic beta cells [26]. Further, PPARγ was associated in genome-wide association studies with type 2 diabetes and obesity [22–24], which might contribute to FABP2 associations. Thus, pancreatic beta cells and adipocytes from FABP2 promoter haplotype B carriers should be used to test this hypothesis.

By means of reporter assays, we measured the rosiglitazone stimulated PPARγ/RXRα induced absolute activities (AA) as well as the induction ratio (IR) between un-stimulated and rosiglitazone stimulated PPARγ/RXRα induced activities of FABP2 haplotype A, B and haplotype chimeras. We found that polymorphism c.-471G>A is involved in determining different AA and IR of FABP2 promoter haplotypes. We have to admit, that the AA of chimera A-471B and the IR of B-471A are not in complete accordance to the corresponding haplotypes B and A, respectively. Therefore, other polymorphism than c.-471G>A or a combination of polymorphisms could be involved in determining different responsiveness of FABP2 promoter haplotypes. Surprisingly, a functional PPARγ binding site was not found in the −471 region but at position −623/−607 of the FABP2 promoter. Based on EMSA, we identified a PPARγ binding site at position −623/−607 of the FABP2 promoter. Activation of −623/−607 promoter mutants by PPARγ/RXRα revealed that this site has limited or no influence on the activity of haplotype A or B, respectively. Thus, the functionality and importance of the −623/−607 PPARγ binding site is questionable. Further PPARγ binding sites seem to be relevant for the induction of FABP2 promoter haplotype activity by PPARγ.

So far, Oct-1 has been described as an important transcription factor for genes involved in immune system regulation [27]. The expression of housekeeping genes encoding histone H2B or snRNA is also dependent on Oct-1 [28]. In the present study we identified FABP2 as a potential target gene of Oct-1. The activity of FABP2 promoter haplotypes is induced by Oct-1 by factor of three. Furthermore, the region of the FABP2 promoter containing the c.-471G>A polymorphism interacts with Oct-1. Interestingly, Oct-1 abolishes the different activities in FABP2 promoter haplotypes induced by rosiglitazone activated PPARγ/RXRα. Thus, high concentration of Oct-1 can override the effect of PPARγ/RXRα on different activities of FABP2 promoter haplotypes. In contrast to the promoter–reporter assays in HeLa cells, we found no effect of Oct-1 on FABP2 mRNA steady level in CaCo2 cells. Thus, our findings seem to be not relevant for intestinal FABP2 expression but could be important for an ectopic expression of FABP2 in PPARγ/RXRα and Oct-1 expressing tissues. Of course, *ex vivo* or *in vivo* approaches are necessary to test this hypothesis. Based on our findings, a functional interaction of PPARγ/RXRα and Oct-1 can be also considered. Supportively, a link between fat metabolism and Oct-1 was revealed by direct interaction between RXR and Oct-1 [29]. More indirectly, PPARγ induces the expression of the Oct-1 [30]. In conclusion, our findings suggest a functional role of PPARγ/RXRα and Oct-1 in the regulation of the FABP2 gene.

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MTTP variants and body mass index, waist circumference and serum cholesterol level: Association analyses in 7582 participants of the KORA study cohort

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ABSTRACT

The microsomal triglyceride transfer protein (MTTP) is a key regulator in the assembly and secretion of chylomicrons and very low density lipoprotein (VLDL) in the intestine and in liver. Associations between MTTP variants and traits of the metabolic syndrome are carried out in relatively small cohorts and are not consistent.

We analysed MTTP polymorphisms in 7582 participants of the KORA study cohort. Seven htSNPs covering a 52 kb region of the MTTP locus and two cSNPs (I128T, H297Q) were selected.

A MTTP haplotype containing the minor allele of H297Q showed a significant decrease of −0.636 (95% CI: −1.226, −0.046; $p = 0.035$) BMI units in females but not in males. In comparison to homozygous H-carriers for the major allele of the MTTP H297Q polymorphism, homozygous Q297Q carriers showed a significant decrease in BMI of −0.425 BMI units (95% CI: −0.74, −0.12; $p = 0.007$), in waist circumference of −0.990 cm (95% CI: 1.74, −0.24; $p = 0.01$) and in total cholesterol of −0.039 mmol/l (95% CI: −0.07, 0; $p = 0.03$). Heterozygous Q-carriers displayed a reduction in BMI of −0.183 BMI unit (95% CI: −0.33, −0.04; $p = 0.012$), in waist circumference of −0.45 cm (95% CI: 0.8, −0.1; $p = 0.01$) and in total cholesterol of −0.103 mmol/l (95% CI: −0.18, −0.03; $p = 0.01$). Gender stratified statistics revealed a significant reduction of −0.657 BMI units (95% CI: −1.14, −0.18; $p = 0.007$), −1.437 cm waist circumference (95% CI: −2.55, −0.32; $p = 0.01$) and −0.052 mmol/l total cholesterol (95% CI: −0.1, −0.01; $p = 0.03$) for females homozygous for the Q297Q polymorphism. Females carrying the Q-allele showed a decrease of −0.259 BMI unit (95% CI: −0.48, −0.04; $p = 0.023$), −0.662 cm waist circumference (95% CI: −1.18, −0.14; $p = 0.01$) and −0.111 mmol/l total cholesterol (95% CI: −0.21, −0.01; $p = 0.03$).

Our association analysis in a large population based study cohort provides evidence that the minor allele of the MTTP H297Q polymorphism is associated with lower BMI, waist circumference and total cholesterol in females but not in males.

Introduction

The microsomal triglyceride transfer protein (MTTP, OMIM accession number: 157147; GenBank: NM_000253.2) gene is located on chromosome 4q24, contains nineteen exons and codes for a 97.4 kDa protein comprising 876 amino acid residues. MTTP is expressed in the liver, the small intestine and the heart. It plays a critical role in the assembly and secretion of very low density lipoproteins (VLDLs) and chylomicrons [1,2]. In the MTTP gene common regulatory (−493G > T, −400A > T, −164T > C) and nonsynonymous (I128T, H297Q) polymorphisms were described [3]. Polymorphisms −493G > T (rs1800591), −400A > T (rs1800803), −164T > C (rs1800804) and I128T (rs3816873) are in complete linkage disequilibrium in Caucasian [3,4]. Several studies indicated that the minor alleles of these polymorphisms are associated with lower LDL levels and protect against risk parameters of the metabolic syndrome [3,5–14]. Opposite or no associations were found in other studies [15–22]. Since these initial association studies were carried out in relatively small cohorts, we analysed MTTP variants regarding their associations to traits of the metabolic syndrome in 7582 participants of the KORA study cohort (S3, S4).

Material and methods

Study population

In the Southern German region of Augsburg including the city of Augsburg and the two surrounding counties, population-based

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surveys of the 25–74 year old population in groups of age range of 5 years were implemented in 1984 as part of the WHO MONICA (multinational MONItoring of trends and determinants in Cardiovascular disease) project and continued since 1996 within the KORA (Kooperative Gesundheitsforschung in der Region Augsburg) platform. The aims of the surveys were to describe the prevalence of common diseases and their risk factors in a representative sample of the adult general population. The current study included the survey of the years 1994–1995 (MONICA S3) including 4856 participants and the survey of the years 1999–2001 (KORA S4) including 4261 participants yielding 9117 recruited participants. The study population of S3 and S4 consisted of all German residents of the Augsburg region who were born between 1920 and 1975 identified through the public record office. More than 99.5% of the participants were Caucasian. The high standards of the WHO MONICA project applied to both surveys. All study participants underwent a standardized interview, a physical examination, and blood withdrawal by trained staff and signed a consent form of the Bavarian Ethics committee and the Ethics committee of the University of Munich. For determination of body weight and height, participants were asked to remove shoes and heavy clothing. The weight measurements were done with a calibrated body weight scale (SECA 709) and were carried out with an accuracy of 0.1 kg. The body height was read to the nearest 0.5 cm on a body height scale. BMI (kg/m^2) was calculated as weight in kilograms divided by height in square meters. The pooled data analysis included only 7582 individuals out of 9117, because complete information on age, sex, BMI and 9 genotypes were not available for the whole group. There was no overlap between the two surveys by design.

Genotyping

Genotyping was performed using a MALDI TOF MS system (Sequenom, Mass EXTEND, San Diego, USA). Haplotype tagging SNPs (htSNPs) with a minor allele frequency >0.1 were selected in order to cover the whole MTTP gene locus (52 kb). The selection was based on Haploview 3.2 [23]. Coding SNPs (cSNPs) rs3816873 (I128T) and rs2306985 (H297Q) were selected based on previous studies and suggested functionality [3,4,13]. Sequences of primers and assay probes are available on request.

Statistical methods

We used a linear regression model with BMI as continuous outcome for both studies combined; the unit change in BMI by the DNA variants was estimated. Haplotype analysis was carried out with the statistical software R (V. 2.3.1. including haplo.stats package) using the haplo.glm procedure. This procedure performs an iterative two-step Estimation-Maximization (EM)-algorithm, with the posterior probabilities of pairs of haplotypes per subject used as weights to update the regression coefficients, and the regression coefficients used to update the posterior probabilities. Haplotypes were included into the regression model all at once except the most common haplotype. Using the expected number of copies of haplotype implies an additive model for each haplotype.

Results

In order to analyse the association between MTTP variants and anthropometric risk parameters, seven htSNPs (rs1503777, rs3811800, rs105763, rs2035816, rs982424, rs2903202, rs1491244) and two nonsynonymous cSNPs (rs3816873, rs2306985) were genotyped in 9117 participants of the KORA study cohort (S3, S4). htSNPs with a minor allele frequency >0.1 were selected in order to cover the whole MTTP gene locus (52 kb). Coding SNPs (cSNPs) rs3816873 (I128T) and rs2306985 (H297Q) were selected based on previous studies and suggested functionality [3,4,13]. Details of the study cohort were published recently [24,25]. In short, the participants were 25–74 years of age, the range of BMI was 14–57 kg/m^2 and approximately 50% were female. The selected htSNPs comprise a 52 kb region of the MTTP locus. The selected cSNPs lead to an amino acid exchange from I128 to T128 (rs3816871) and H297 to Q297 (rs2306985), respectively. The two other known cSNPs Q95H and Q244E of the MTTP gene were not genotyped because of their low frequencies. Haplotypes were computed with the nine htSNPs and cSNPs. As shown in Table 1, we identified nine haplotypes with frequencies between 2.7 and 53.0%. The minor allele frequencies of I128T and H297Q were 27.2 and 35.96%, respectively. Next, we analyzed associations between MTTP haplotypes (Table 1) or single SNPs (Table 2) with BMI, waist circumference and total cholesterol by linear regression analysis. Gender-stratified statistics were also done.

As shown in Table 1, haplotype VII showed a significant decrease of −0.636 (95% CI: −1.226, −0.046; p = 0.035) BMI units in females but not in males in comparison to the major haplotype I. Other haplotypes were not significantly associated with BMI. With the exception of H297Q, we found no appreciable association of BMI, waist circumference and cholesterol levels with single MTTP SNPs. Compared with homozygous H-carriers (H297H) for the major allele of the MTTP H297Q polymorphism, homozygous Q297Q carriers showed a significant decrease in BMI of −0.425 BMI units (95% CI: −0.74, −0.12; p = 0.007) and in waist circumference of −0.990 cm (95% CI: 1.74, −0.24; p = 0.01) in the whole study group (Table 2). Carriers of the minor allele of MTTP H297Q SNP showed a significant reduction in BMI of −0.183 BMI unit (95% CI: −0.33, −0.04; p = 0.012) and waist circumference of

Table 1
Associations of MTTP haplotypes with BMI as continuous variable

Haplotypes	Frequencies				Unit change in BMI in kg/m^2 [95% confidence interval] (*p* values)		
	All (%)	Men (%)	Women (%)		All[a]	Men[b]	Women[b]
I, 212212112	53.0	53.7	52.3				
II, 212112212	13.5	13.3	13.7	I vs II	−0.143 [−0.362, 0.077] (0.204)	−0.023 [−0.248, 0.293] (0.87)	−0.305 [−0.624, 0.014] (0.061)
III, 121112112	11.0	10.9	11.1	I vs III	0.144 [−0.092, 0.379] (0.232)	0.050 [−0.237, 0.337] (0.733)	0.265 [−0.084, 0.614] (0.136)
IV, 221122221	5.9	5.8	5.9	I vs IV	−0.090 [−0.402, 0.221] (0.569)	−0.044 [−0.429, 0.342] (0.825)	−0.168 [−0.627, 0.291] (0.472)
V, 121112221	3.8	3.7	3.9	I vs V	−0.275 [−0.65, 0.1] (0.151)	−0.172 [−0.642, 0.298] (0.473)	−0.299 [−0.840, 0.242] (0.278)
VI, 221122222	3.2	3.2	3.1	I vs VI	0.009 [−0.403, 0.420] (0.967)	−0.134 [−0.631, 0.363] (0.596)	0.156 [−0.426, 0.738] (0.598)
VII, 222111212	3.1	2.9	3.3	I vs VII	−0.379 [−0.794, 0.036] (0.073)	−0.077 [−0.595, 0.442] (0.771)	**−0.636 [−1.226, −0.046] (0.035)**
VIII, 222112212	2.8	2.7	2.9	I vs VIII	−0.106 [−0.54, 0.328] (0.631)	−0.096 [−0.629, 0.438] (0.725)	−0.114 [−0.709, 0.481] (0.708)
IX, 122111212	2.7	2.7	2.7	I vs IX	−0.33 [−0.772, 0.113] (0.144)	−0.462 [−1.002, 0.078] (0.094)	−0.221 [−0.804, 0.362] (0.458)

Results are the beta estimate (linear regression) with 95% confidence interval and p values. Haplotype designation: the allele with the higher prevalence is denoted with a, the minor allele with b. The order of polymorphisms corresponds to their position in the 5′ to 3′ direction of the gene: rs1503777, rs3811800, rs3816873 (I128T), rs105763, rs2035816, rs982424, rs2306985 (H297Q), rs2903202, rs1491244. The main results are in bold.

Table 2
Associations of MTTP H297Q genotypes with BMI, waist circumference and total cholesterol as continuous variable

Genotypes	Frequencies			Unit change in BMI in kg/m^2 [95% confidence interval] (*p* values)		
	All(*n*)	Men(*n*)	Women(*n*)	All	Men	Women
BMI						
HH	3115	1585	1530			
HQ	3481	1716	1765	−0.106 [−0.32, 0.1] (0.32)	−0.143 [−0.4, 0.11] (0.27)	−0.072 [−0.4, 0.26] (0.67)
QQ	986	479	507	**−0.425 [−0.74, −0.12] (0.007)**	−0.200 [−0.58, 0.18] (0.31)	**−0.657 [−1.14, −0.18] (0.007)**
Q[a]	4467	2195	2295	**−0.183 [−0.33, −0.04] (0.012)**	−0,112 [−0.29, 0.06] (0.21)	**−0.259 [−0.48, −0.04] (0.023)**
Waist						
HH	3120	1589	1531			
HQ	3488	1720	1768	−0.334 [−0.84, 0.17] (0.20)	−0.162 [−0.83, 0.51] (0.64)	−0.509 [−1.27, 0.25] (0.19)
QQ	989	483	506	**−0.990 [−1.74, −0.24] (0.01)**	−0.561 [−1.57, 0.44] (0.27)	**−1.437 [−2.55, −0.32] (0.01)**
Q[a]	4477	2203	2274	**−0.450 [−0.8, −0.1] (0.01)**	−0.246 [−0.71, 0.22] (0.30)	**−0,662 [−1.18, −0.14] (0.01)**
Total cholesterol						
HH	3132	1590	1542			
HQ	3507	1723	1784	−0.007 [−0.06, 0.04] (0.78)	0.03 [−0.05, 0.11] (0.45)	−0.046 [−0.11, 0.02] (0.21)
QQ	994	484	510	**−0.103 [−0.18, −0.03] (0.01)**	−0.103 [−0.22, 0.01] (0.08)	**−0,111 [−0.21, −0.01] (0.03)**
Q[a]	4501	2207	2294	**−0.039 [−0.07, 0] (0.03)**	−0.028 [−0.08, 0.02] (0.30)	**−0,052 [−0.1, −0.01] (0.03)**

Results are the beta estimate (linear regression) with 95% confidence interval and *p* values. The main results are in bold.
[a] additive model.

−0.45 cm (95% CI: 0.8, −0.1; p = 0.01). Gender stratified statistics revealed a significant reduction of −0.657 BMI units (95% CI: −1.14, −0.18; p = 0.007) and waist circumference of −1.437 cm (95% CI: −2.55, −0.32; p = 0.01) for females homozygous for the Q297Q polymorphism. Females carrying the Q-allele showed a decrease of −0.259 BMI unit (95% CI: −0.48, −0.04; p = 0.023) and waist circumference of −0.662 cm (95% CI: −1.18, −0.14; p = 0.01). These associations were not observed in males.

Association of total cholesterol with the Q-allele showed a reduction of −0.0391 mmol/l (95% CI: −0.07, 0; *p* = 0.03) (Table 2). Homozygous carriers displayed a total cholesterol reduction of −0.103 mmol/l (95% CI: −0.18, −0.03; p = 0.01). After stratification for gender, female but not male Q-allele carrier had a −0.052 mmol/l (95% CI: −0.1, −0.01; p = 0.03) lower total cholesterol. Female homozygous carriers exhibited a total cholesterol reduction of −0.111 mmol/l (95% CI: −0.21, −0.01; p = 0.03). No association between the H297Q polymorphism and HDL- or LDL-cholesterol was observed (data not shown).

Discussion

MTTP is a key regulator of chylomicron and VLDL assembly and secretion in the intestine and in liver. Loss of function mutations in the MTTP gene lead to a truncated protein and cause abetalipoproteinemia [26], fat malabsorption [27], hypercholesterolemia [6] and hepatic steatosis [28]. Functional studies of MTTP promoter haplotypes comprising SNPs −493G > T (rs1800591), −400A > T (rs1800803) and −164T > C (rs1800804) revealed two fold lower activity of the major haplotype −493G/−400A/−164T in comparison to the minor haplotype −493T/−400T/−164C [4]. Further, the major allele of MTTP I128T confers increased functionality of the protein [29]. The functional promoter and I128T polymorphisms are in complete linkage disequilibrium: Therefore, the resulting two functional MTTP haplotypes A and B contain combinations of a high (low) promoter activity and low (high) protein functionality. The overall consequences of this opposite effects are not known. However, studies of polymorphism −493G > T which covered the two functional haplotypes showed positive, negative, or no associations with traits of the metabolic syndrome [3,5–14].

Here we analyzed seven htSNPs as well as two common cSNP in the MTTP gene for associations with BMI, waits circumference, and total cholesterol in a large population based study cohort comprising 7582 participants [24,25]. The selected SNPs cover a 52 kb MTTP gene region and contain the functional haplotypes as well as cSNP H297Q. We found no association between BMI, waist circumference, or total cholesterol and I128T which represent the functional haplotypes A and B. Our finding supports the idea that the overall effects of these haplotypes are neutral. To our surprise, the largely unnoted cSNP H297Q [3] showed association with BMI, waist circumference and total cholesterol, whereupon the minor Q-allele carrier showed a general reducing effect. Thus, it seems to be fruitful to reanalyse published results as shown for the association between MTTP H297Q SNP and traits of the metabolic syndrome. An exchange of His to Gln at position 297 results in a change of a weakly basic to a neutral side chain making this side more hydrophobic. Amino acid residue 297 is located in a domain relevant for lipid binding, suggesting functionality of H297Q SNP. Of course, functional studies are necessary to test this hypothesis.

A direct link between MTTP functionality and BMI or waist circumference is not apparent. However an interaction with total cholesterol is plausible, since MTTP is relevant for chylomicron and VLDL maturation via apoB binding. Accordingly, involvement of the MTTP gene in the development of fat malabsorption, hepatic steatosis and insulin resistance were demonstrated in patients with loss of function mutations in the MTTP gene [3,27] as well as in MTTP knock-out mice [28,30]. Associations with the MTTP H297Q polymorphism were only found in females. A sex-specific response of the MTTP −493G > T to dietary lipid changes was described recently [31]. MTTP and apoB are building a complex, therefore protein interactions have to be considered. It was shown that apoB mRNA and protein levels are higher in females [32,33]. Accordingly, altered MTTP activity by H297Q polymorphism may have more pronounced effects in females than in males resulting in a stronger phenotype change.

In conclusion, our association analysis in a large population based study cohort provides evidence that the minor allele of the MTTP H297Q polymorphism is associated with lower BMI, waist circumference and total cholesterol in females but not in males.

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Association analysis between the PTGES2 R298H polymorphism and body mass index in 8079 participants of the KORA study cohort

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Association analysis between the PTGES2 R298H polymorphism and body mass index in 8079 participants of the KORA study cohort

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Abbreviations: PTGES2: Prostaglandin E2 Synthase 2; htSNPs: Haplotype Single Nucleotide Polymorphisms; KORA: Kooperative Gesundheitsforschung in der Region Augsburg; BMI: Body Mass Index; PGE_2: Prostaglandin E_2; WHO MONICA: multinational MONItoring of trends and determinants in Cardiovascular disease; PPARγ: peroxisome proliferator-activated receptor gamma

Abstract

Context: The H-allele of the R298H polymorphism in the Prostaglandin E Synthase 2 (PTGES2) gene was associated with lower risk of diabetes type 2.

Aim: In order to explore the association between the PTGES2 R298H SNP and BMI, we analysed the R298H SNP (rs13283456) and three htSNPs (rs884115, rs10987883, rs4837240) covering a 20 kb gene region in population based surveys of the KORA study cohort with 8079 participants.

Results: A statistically significant difference in BMI between the heterozygous PTGES2 R298H genotype and the homozygous R/R genotype was found in males but not in females. Males with the R/H genotype showed a decrease in BMI of -0.30 BMI units (95% CI: -0.55, -0.04, p=0.02) in comparison to R/R males. A haplotype comprising the minor allele of PTGES2 R298H showed a significant decrease of -0.23 BMI units in males (-0.45, -0.02; p=0.04) but not in females. Other haplotypes and single htSNPs were not significantly associated with BMI.

Conclusion: We found a marginal but significant influence of the PTGES2 298H SNP on BMI in a large population based study.

Introduction

Prostaglandin E2 (PGE_2) is produced in diverse tissues and is involved in a number of biological processes including inhibition of lipolysis, induction of hypertrophy of adipose tissue and leptin secretion (Gaskins et al. 1989; Fain et al. 2001; Serhan and Levy 2003; Murakami and Kudo 2004). PGE_2 is synthesized by PGE_2 synthases (PTGES) in concert with phospholipase A2 and cyclooxygenases. Three PTGES isoforms are known. The microsomal PTGES (PTGES1) is mainly involved in inflammation, whereas the role of the cytosolic PTGES is uncertain (Gaskins et al. 1989; Murakami and Kudo 2004). The membrane-associated PTGES2 is mainly expressed in tissues such as adipocytes with low PTGES1 levels and is not inducible by inflammation (Tanikawa et al. 2002). The PTGES2 (NM_025072) gene maps to the chromosomal band 9q34.11, which was significantly associated with body weight (Wilson et al. 1991). Recently, we showed in three independent study populations that the PTGES2 R298H (rs13283456) polymorphism is associated with type 2 diabetes and related traits (Fisher et al. 2007; Lindner et al. 2007; Nitz et al. 2007). Whether the rare H-allele contributes to body mass index (BMI) remained questionable. Therefore, we analyzed the PTGES2 R298H SNP with BMI in large (n=8079) populations based surveys (S3, S4) of the KORA study cohort.

Material and Methods

Study population

In the Southern German region of Augsburg including the city of Augsburg and the two surrounding counties, population-based surveys of the 25-74 year old population in groups of age range of 5 years were implemented in 1984 as part of the WHO MONICA (multinational MONItoring of trends and determinants in Cardiovascular disease) project and continued since 1996 within the KORA (Kooperative Gesundheitsforschung in der Region Augsburg) platform. The aims of the surveys were to describe the prevalence of common diseases and their risk factors in a representative sample of the adult general population. The current study included the survey of the years 1994-1995 (MONICA S3) including 4856 participants and the survey of the years 1999-2001 (KORA S4) including 4261

participants yielding 9117 recruited participants. The study population of S3 and S4 consisted of all German residents of the Augsburg region who were born between 1920 and 1975 identified through the public record office. More than 99.5% of the participants were Caucasian. The high standards of the WHO MONICA project applied to both surveys. All study participants underwent a standardized interview, a physical examination, and blood withdrawal by trained staff and signed a consent form of the Bavarian Ethics committee and the Ethics committee of the University of Munich. For determination of body weight and height, participants were asked to remove shoes and heavy clothing. The weight measurements were done with a calibrated body weight scale (SECA 709) and were carried out with an accuracy of 0.1 kg. The body height was read to the nearest 0.5 cm on a body height scale. BMI (kg/m²) was calculated as weight in kilograms divided by height in square meters. The pooled data analysis included 8079 individuals with complete information on age, sex, BMI and genotypes. There was no overlap between the two surveys by design.

Genotyping

Genotyping was performed using a MALDI TOF MS system (Sequenom, Mass EXTEND, San Diego, USA). Sequence information of the *PTGES2* gene (ID: 80142) was derived from Genbank (www.ncbi.nlm.nih.gov). As described (Nitz et al. 2007), the R298H SNP (rs13283456) was identified by re-sequencing and htSNPs were selected by analysing a 20 kb region comprising *PTGES2* (Chrom 9: pos. 127.952.200-127.972.200) using Haploview 3.2. HtSNPs with a minor allele frequency >0.1 (rs10987883, rs4837240, rs884115) were obtained from CEPH HapMap data release 21 (www.hapmap.org). Sequences of primers and assay probes are available on request.

Statistical methods

We used a linear regression model with BMI as continuous outcome for both studies combined; the unit change in BMI by the DNA variants was estimated. Haplotype analysis was carried out with the statistical software R (V. 2.3.1. including haplo.stats package) using the haplo.glm procedure. This procedure performs an iterative two-step Estimation-Maximization (EM)-algorithm, with the posterior probabilities of pairs of haplotypes per subject used as weights to update the regression coefficients,

and the regression coefficients used to update the posterior probabilities. Haplotypes were included into the regression model all at once except the most common haplotype. Using the expected number of copies of haplotype implies an additive model for each haplotype.

Results

A total of 8079 subjects of the KORA study cohort (S3, S4) were genotyped for the PTGES2 R298H SNP. Three htSNPs (rs884115, rs10987883, rs4837240) covering a 20 kb region of the PTGES2 gene were also genotyped. The participants were 25-74 years of age, approximately half were male and the range of BMI was 14-57 kg/m^2. More details of the study cohort were described elsewhere (Heid et al. 2005). Gender-stratified statistics of the BMI and their associations to the PTGES2 R298H SNP as well as haplotypes are provided in table 1. For the three htSNPs, we found no significant associations to BMI (not shown). In females, no significant association between PTGES2 genotypes and BMI was observed. We found a statistically significant difference in BMI between heterozygous PTGES2 R298H males and males homozygous for the major allele (R/R). R/H males showed a decrease in BMI of -0.30 BMI units (95% CI: -0.55, -0.04, p= 0.02) in comparison to R/R males. This result was congruent with the association between BMI and haplotype II, which covered the minor allele of R298H. This haplotype showed a significant decrease of -0.19 BMI units (-0.37, -0.01; p=0.04) for the hole study population and -0.23 BMI units (-0.45, -0.02; p=0.04) in males. There was no association with haplotypes detected in females. Other haplotypes were not significantly associated with BMI. Further, no linkage of the R298H polymorphism with obesity status (BMI>30) or waist to hip ratio was observed (not shown).

Discussion

The metabolite of PTGES2 PGE_2 possesses anti-lipolytic activity, induces hypertrophy in adipose tissue and is involved in secretion of leptin (Richelsen 1987; Gaskins et al. 1989; Richelsen 1991; Richelsen et al. 1991; Fain et al. 2000; Fain et al. 2001; Serhan and Levy 2003; Murakami and Kudo

2004). These characteristics, the linkage of the 9q34.11 PTGES2 gene region with body weight (Wilson et al. 1991) and the association between PTGES2 R298H SNP and traits of the metabolic syndrome (Fisher et al. 2007; Lindner et al. 2007; Nitz et al. 2007) raise the PTGES2 gene as a candidate gene for obesity. On the other hand, genome-wide association studies did not ennoble PTGES2 as a prime candidate for obesity (Herbert et al. 2006; Frayling et al. 2007; Scuteri et al. 2007; Liu et al. 2008). In order to unravel the influence of genetic variance of PTGES2 on body weight, we used a large population based study cohort comprising 8079 participants. Based on this large association study, a reduced BMI of -0.30 BMI units was observed in males heterozygous for the PTGES2 R298H SNP. A haplotype comprising the minor allele of R298H was also associated with a reduced BMI. Thus, the PTGES2 298H allele leads to a slight reduction of the BMI in males. A protective effect of the H-allele was also described for diabetes type 2, impaired glucose tolerance, fasting and postprandial insulin levels and HOMA indices for ß-cell function and insulin resistance (Herbert et al. 2006; Frayling et al. 2007; Scuteri et al. 2007; Liu et al. 2008). The BMI lowering effect of the His-allele was solely observed in heterozygous but not in homozygous males. Although this specific association remain ambiguous, based on functional considerations suggestive hypotheses can be formulated. We assumed that the heterozygous genotype leads to a slight reduction in overall PTGES2 function causing a reduced PGE_2 level. This could result in a lower BMI, because PGE_2 possesses anti-lipolytic activity and induces adipocyte hypertrophy (Richelsen 1987; Richelsen 1991; Richelsen et al. 1991). An even stronger reduction of PGE_2 levels in homozygous carriers of the PTGES2 298H allele may induces compensatory up-regulation of other PTGES2 enzymes. The reason for a specific protection of the PTGES2 298H allele in males might be caused by gender-specific PGE_2 levels which might be regulation by transcriptional repression of PTGES genes via PPARγ (Mendez and LaPointe 2003) since PPARγ is regulated in a sex specific manner (Ciana et al. 2007). However, this polymorphism has only a small impact on overall BMI status, although, the H-allele may contribute to a risk-reduction of diabetes type 2 and related traits.

In future functional studies it would be interesting to analyse the gender specific connections between lipolysis, BMI and the PTGES2 R298H. In conclusion, our results in a large population based study cohort provide evidence that the PTGES2 298H allele is associated with lower BMI.

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Author Disclosure Statement

There is no conflict of interest that any author should disclose. A. F. was responsible for collecting and evaluation of the data, genotyping and writing various parts of the manuscript. H.G., M.B., C.G., I.B. and I.H. were involved in data collection and data management. H.-E. W., F.D. and T.I. were the principle investigators, responsible for the study design and assisted in manuscript preparation. No competing financial interests exist.

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Table 1. Frequencies of PTGES2 R298H genotypes (R/R, R/H, H/H) and haplotypes and their associations with BMI as continuous variable via linear regression

	Frequencies			Unit change in BMI in kg/m^2 [95 % confidence interval] (p values)		
	All	Men	Women	All	Men	Women
Genotypes	n	n	n	*Comparison*		
all	8079	4013	4066			
R/R	5203	2620	2583			
R/H	2549	1228	1321	R/R vs R/H	-0.17 [-0.37, 0.04] (0.11)	**-0.30 [-0.55, -0.04] (0.02)**	-0.03 [-0.35, 0.29] (0.87)
H/H	327	165	162	R/R vs H/H	-0.15 [-0.64, 0.33] (0.54)	0.01 [-0.58, 0.60] (0.97)	-0.37 [-1.14, 0.39] (0.34)
R/H+H/H	2876	1393	1483	R/R vs H-carrier[1]	-0.13 [-0.3, 0.04] (0.13)	-0.17 [-0.38, 0.03] (0.10)	-0.09 [-0.35, 0.17] (0.50)
Haplotypes	%	%	%			
I, 1222	63.1	63.6	62.6			
II, 1212	19.7	19.3	20.2	I vs II	**-0.19 [-0.37, -0.01] (0.04)**	**-0.23 [-0.45, -0.02] (0.04)**	-0.13 [-0.41, 0.14] (0.34)
III, 2121	10.6	10.3	11.0	I vs III	-0.09 [-0.31, 0.14] (0.46)	-0.18 [-0.46, 0.10] (0.20)	0.06 [-0.30, 0.41] (0.75)
IV, 2222	3.5	3.5	3.5	I vs IV	0.01 [-0.37, 0.40] (0.95)	-0.14 [-0.61, 0.34] (0.57)	0.09 [-0.52, 0.69] (0.78)

Results are the beta estimate (linear regression) with 95 % confidence interval and p values adjusted for age. [1] additive model. Haplotype designation: the allele with the higher prevalence is denoted with 1, the minor allele with 2. The order of polymorphisms corresponds to their position in the 5´ to 3´ direction of the gene: rs884115, rs13283456 (R298H), rs10987883, rs4837240. The main results are in bold.

Lebenslauf

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Erklärung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertation mit dem Titel

„Functional and association analysis of polymorphisms in selected genes of fat metabolism“

selbständig und ohne unerlaubte Hilfe angefertigt habe und dass ich die Arbeit noch keinem anderen Fachbereich bzw. noch keiner anderen Fakultät vorlegt habe.

Mike Böhme

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